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PHYSIOLOGICAL ZOÖLOGY

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CRITICAL STAGES OF HAIR DEVELOPMENT AND PIGMENTATION IN THE MOUSE

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For a thorough study of the skin under normal and experimental conditions and in cancerous and precancerous phases, it is necessary to understand normal growth cycles and age variations in all parts of the skin. In this paper, stages in the normal hair-development cycle are under consideration. Dry (1926) described in the mouse the basic general stages of hair development and offered the terms "anagen" (1–17 days) for active proliferation; "catagen" (17–19 days) for cessation of proliferation and pigment deposition and for formation of the club and germ; and "telogen" for the resting stage. Butcher (1934; Johnson, Butcher, and Bevelander, 1945) emphasized that in the rat there are cycles of activity and rest. For experimental use, at least, we feel that greater detail in these stages is of value, and Dry's anagen stage has been divided into six rather precise and recognizable substages.

Plucking of club hairs is known to initiate growth of the succeeding hair generation (Collins, 1918; David, 1934; Schwanitz, 1938; Chase, 1946, 1949*a*, *b*). Control of hair stage in terms of number of days after plucking has thus been possible, and treatments have been applied at such known stages. Since the initiation of growth due to plucking varies by as much as 2–3 days and since individual follicles vary considerably in the early stages, it is desirable to have more precise morphological information. Furthermore, where natural waves of growth are to be allowed, it is necessary to be able to identify the stages by accurate means. Timing by age of young mice has been employed (Suntzeff, Carruthers, Cowdry, 1947) but is clearly ineffectual because individual follicles, even from the dorsum of a newborn mouse, vary widely. In such a newborn mouse some follicles are in an advanced stage comparable to that reached 5 days after plucking, whereas some follicles are just invaginating or are yet to invaginate (cf. Gibbs, 1941).

Some intermediate stages in the hair cycle have been described for the rat by Butcher (1934) and Taylor (1949), for the rabbit by Schwanitz (1938) and Lübnow (1939), and for the mouse by Kaliss (1942). A more detailed description for the whole hair cycle and a discussion of the dynamics of hair growth are included in the present paper. The critical stages of hair and pigment development can serve partly in identifying stages

[1] This work was supported by a grant-in-aid from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

and partly in understanding the effects of various treatments at particular stages. Phases of normal and abnormal development in other portions of the skin system can be related to hair and pigment phases when these phases become better defined and understood. A beginning has been made in the work of Parnell (1949) on the functional cycles of the sebaceous glands and of Montagna and Chase (1950) on redifferentiation of the sebaceous glands.

MATERIALS AND METHODS

Skin from mice of the C57 Black strain was used for timed samples. For a normal young series, pieces of skin were taken from the mid-dorsum and mid-ventrum. Specimens were taken for each day of age from newborn to 19 days. In older mice, areas on the dorsum were plucked of club hairs to initiate growth of the next hair generation, then pieces of skin were taken representing daily stages from 0 to 19 days and at 21, 22, and 26 days. For comparison, some stages were used from albino and piebald mice.

Portions of each tissue, fixed in formol-calcium, were mounted in gelatin and were sectioned with the freezing microtome at 5 and occasionally 10 μ. These sections were unstained. For comparison, other portions were imbedded in paraffin, and sections were stained with haematoxylin and eosin or with a modified Masson's trichrome technique. To emphasize the mitotic activity of certain stages, colchicine-treated animals were used as supplemental material. Colchicine in the concentration of 0.1 mg/30 gm body weight was injected intraperitioneally, and tissues were taken $9\frac{1}{2}$ hours later.

Most of the observations were made on the unstained frozen sections under the Spencer phase-contrast microscope. An oil-immersion, B minus, contrast-low objective was employed primarily. The oil-immersion, dark contrast-medium objective (A minus) served for verification of certain details. Pigment cells were seen most clearly in frozen sections studied under the B minus objective.

OBSERVATIONS

The anagen stage (Dry, 1926) of the hair cycle is the phase of active proliferation. During this period, however, no cell divisions occur in the dermal papilla. For experimental studies on the skin, this general anagen stage can be subdivided into six critical steps which are readily recognized. The regenerating hair and the first hair growth of the young mouse, after the initial invagination, follow identical patterns of development. The basic component in hair development is the germ or germ plate, which is generally a single layer of relatively large cells more or less continuous with cells of the external sheath (Figs. 3 and 11). The six anagen substages, the catagen, and the telogen are described below, primarily in terms of this germ, the bulb it forms, and the hair shaft and inner sheath, formed, in turn, by the bulb.

Anagen I.—Cells of the germ plate go into cell division. In new growth, induced by plucking, this stage occurs generally about 1–2 days after the plucking stimulus. In a new follicle which is invaginating from the skin surface, cell divisions are occurring throughout the walls of the invagination, i.e., the external sheath which is continuous with the basal layer of the Malpighian layer of skin. The lower end of this invagination soon has the greatest activity, however, and is essentially the germ.

Anagen II.—There is a downgrowth of the germ plate around the ball of

papillary cells. The inverted cone of epidermal cells around the papilla cells is the beginning of the papilla cavity (Fig. 4). At the same time a thin keratinized dome appears just above this layer of epidermal cells. This dome is the beginning of the internal sheath. The base of the follicle, demarcated by the connective-tissue sheath immediately below the papilla cells, is characteristically about 250 μ, or at the "resting" level, below the skin surface at this time. Such a stage is attained about 1 day following the initiation of mitotic activity in the germ, or generally about 2–3 days after plucking.

Anagen III.—In this stage the internal sheath is no longer a dome but is pointed and begins to project upward through the tube of the external sheath (Fig. 7). The papilla cavity becomes constricted at the base, but it is still wide and rounded at the upper end and middle (Fig. 6). The original germ plate has now proliferated to such an extent that there is a bulb of matrix cells. The first pigmentary dendritic cells appear in the bulb (Fig. 5). They are located, for the most part, among the cells of the basal layer and specifically in that portion of the basal layer near the apex and upper third of the papillary cavity (Fig. 6). These cells with fine, dispersed pigment granules have processes extending usually two cell layers laterally from the cavity in the small zigzag follicles and up to five layers in the larger follicles. The dendritic cells, which become melanogenic at this time, are distinct from the clumped pigment remnants which frequently are observed in the germ, in the external sheath, and even in the papilla of resting and early regenerating stages. These are remains from the previous hair generation. By continued proliferation in the external sheath, the base of the whole follicle stretches downward during this period. The Anagen III stage is found 3 and 4 days after plucking.

Anagen IV.—The hair shaft is clearly defined, having both cortex and medulla. The cuticle of the inner sheath and the adjoining cuticle of the shaft are discernible. The tip of the hair, which is entirely cortex and cuticle, is at about the level of the base of the sebaceous gland. A long and narrowed papillary cavity is characteristic (Fig. 8). Pigment granules are now found in the inner matrix cells of the upper third or half of the bulb (Fig. 9). These pigmented matrix cells appear as an inverted cone within the bulb. The base of the follicle is now typically at least 500 μ below the skin surface and does not usually go any deeper. Such a stage is observed 6 days after plucking.

Anagen V.—At this stage the hair shaft has passed beyond the duct of the sebaceous gland, where the original vanguard internal sheath stops, and the tip is at the skin surface. The cavity of the papilla has become more narrow, even compressed, and is frequently acentric (Fig. 10). The bulb is at its maximum diameter. Under gross observation the skin now appears dark. This is comparable to the stage reached 8 days after plucking.

Anagen VI.—Growth continues for the next 8 or 9 days with no change in the form of the bulb. The rate of growth is approximately 1 mm. of hair shaft per day. The critical formative stages have already occurred; this is simply the stage of rapid growth, analogous to foetal growth.

Catagen.—This stage (Dry, 1926) is characterized by the cessation of matrix-cell proliferation in the bulb. The pigmentary dendritic cells usually cease producing pigment granules at this time or shortly before. Generally, no further

cells enter the shaft as medulla. The club is formed and consists of a hollow brush of keratinized cells at the lower end of the compact cortex. The germ of the next hair generation is set apart as a layer of cells below the club. The papilla cells are found as a rounded-up "ball" of cells. Pigment clumps and degenerate pigment cells are observed usually in the germ region, with occasionally some among the papilla cells. The club region shrinks and moves upward toward the level of the base of the sebaceous gland. The final length of protruding hair is reached in this stage. Also at this time the base of the follicle moves distally to the resting position about 250 μ below the skin surface. Most of the catagen-stage processes are of about 2 days' duration.

Telogen.—By approximately 19 days after plucking, the telogen or "resting" stage is reached (Fig. 11). In young animals, especially for the first three hair generations, the telogen may last 10 days or less. In older animals the telogen may persist for months in a particular region before the new wave starts.

DISCUSSION

The detailed observations of hair growth given in this paper might lead to a greater understanding of hair growth if one considered the homology between the development of the hair and that of the epidermis. The hair follicle may be considered as a device whereby epidermal keratinized material is concentrated. The location and function of pigmentary dendritic cells and papillary cells, as well as the shape of the bulb, become more intelligible from this point of view. During the development of the animal the first indication of a follicle is an invagination of the basal cells of the Malpighian layer of the surface epidermis. These cells give rise to the external sheath cells. Pigmentary dendritic cells (Masson's "melanoblasts") are found at the basement membrane of the surface epidermis, with cell bodies lying between cells of the Malpighian basal layer (Fig. 1). These pigment cells "inoculate" Malpighian cells by way of the dendrites (Billingham and Medewar, 1948; Masson, 1948; Zimmerman and Cornbleet, 1948; Billingham, 1949; Medewar, 1950, in the skin epidermis. Riehl, 1884; Willier, 1942; Chase and Smith, 1949, 1950, in the hair bulb). Such pigment cells are readily seen, especially in skin epidermis of young mice (newborn to 10 days). In the case of older mice, however, these cells never become very active melanogenically except under certain conditions. Some of these "melanoblasts," usually unpigmented at first unless treated with the dopa reagent (Fig. 2), are included in the original follicular invagination (Chase and Smith, 1950). Strongly melanogenic cells, identical in every way with pigmentary dendritic cells of the bulb, are found on the surface of the sebaceous glands, but they never "inoculate" the gland cells. Dermal melanophores are present in the mouse, but no direct evidence is yet available that these cells, with their larger pigment granules, invade the epidermis of the follicle or are in any direct way related to the epidermal melanophores or melanoblasts (cf. Masson, 1948).

The initial source of pigment cells is presumably the neural crest (Du Shane, 1935, 1944; Rawles, 1940, 1947). By grafting embryonic skin and neural-crest material of C57 Black mice to the embryonic coelom of the White Leghorn fowl, Rawles (1947) has shown that pigment is not autonomous in hair but requires neural-crest material in the graft. It has been suggested from the results of X-raying (Chase, 1949*a;* Chase and Smith, 1950) that these potential

pigment cells first enter the embryonic skin epidermis and then are incorporated into the follicular invagination. Pigmentation of the hair is due to pigment granules within the cytoplasm of certain matrix cells. These cells modify the degree and type of melanization of these sites, depending on the genotype and "nutritional" conditions involved. Some authors have suggested that these cytoplasmic sites are of intrinsic origin (Danneel and Lübnow, 1936; Lübnow, 1939; Kaliss, 1942), but it seems more probable now (Masson, 1948; Chase and Rauch, 1950; Chase and Smith, 1950) that the matrix cells receive these "sites" from pigmentary dendritic cells somewhat in the manner described by Riehl (1884). The embryological evidence of Rawles (1947) that mouse pigment is nonautonomous in the matrix cells of the hair is especially conclusive.

Shortly after the initiation of the invagination of the follicle, a dimple forms at its enlarged lower end. As the growth proceeds downward, this dimple expands downward to form a papilla cavity, which surrounds a few dermal cells. With the formation of the papilla cavity and the inclosure of the papilla cells, the hair bulb is established. The bulb is the site of mitotic activity in hair proliferation. The bulb is simply an extension of the epidermal Malpighian layer, folded down over a core of dermal cells. If a bulb were flattened, it would be equivalent to a circle of surface epidermis 160 μ in diameter. This may produce a hair 20 μ in diameter. Of course, part of the bulb gives rise to cornified internal-sheath material and not to the shaft. Nevertheless, compared with the approximate one-to-one relation of corneum to Malpighian layer in mouse surface epidermis, there is a marked concentration of cornified material with relation to the Malpighian (bulb) layer producing it.

At first, much of the activity is an increase in surface, that is, a lengthening of the bulb. The basal layer grows down around the papilla. Comparable cells comprise the external sheath, and divisions there cause the whole follicle to elongate. In the bulb, cell divisions next become predominantly lateral or perpendicular to the papilla cavity—in essence, perpendicular to the surface. Maximum mitotic activity is present in these matrix cells of the bulb, especially the cells of the basal layer at the lower end and their immediate descendants. As the descendant cells are forced outward and upward in an arc, mitoses become less frequent, and keratinization progresses. The cells become part of the hair cortex, cuticle, inner-sheath cuticle, and inner sheath proper. The extensive proliferation perpendicular to the basal layer apparently compresses the papilla cavity to a very small diameter.

The original plate, or germ, of basal cells, which extends downward to form the papilla cavity, is also associated with the first keratinized material. A dome of keratinized cells forms above this layer and constitutes the first production of the internal sheath. The rapid change to a pointed, elongated sheath is the result of proliferation and change in position of this basal layer (Figs. 4 and 7). Later contributions to the sheath are from the matrix cells arising from the lower portion of the bulb.

Pigmentary dendritic cells, as seen through the special objective on the phase microscope, are interspersed with these cells of the original plate or germ. A few cells are to be seen occasionally in less specialized regions of the external sheath. Just as in human skin (Masson, 1948; Zimmerman and Cornbleet, 1948), these cells supply basal and overlying

"Malpighian" cells with granules. Only two layers of cells become pigmented in zigzag or "underfur" follicles. Those beyond, which form the two cuticles and the internal sheath, are generally not pigmented. At the end of a cycle of growth some degenerate pigment cells or pigment clumps are seen in the germ region and among the papilla cells (cf. with human skin, Masson, 1948). No evidence is available of active pigment cells entering the epidermis from the papilla in this material.

There is a certain amount of stratification in the matrix cells (Fig. 8), and it appears that the inner sheath is derived from the lowest level, the two cuticles from the next levels, and the cortex from the level at the edge of the pigmented zone. The medulla may arise from similar cells immediately above the latter (Lübnow, 1939). The cortical and medullary cells are the cells which receive pigment.

At the end of active proliferation the catagen phenomena take place. At this time or before, pigment deposition ceases, and the shaft has either no medulla or, in its place, a narrow canal with no cells. The formation of the club and the setting-aside of the hair germ of the next generation are the principal features of the catagen (Dry, 1926). The dynamics of this stage are not known, but it is suggestive that the changes are directly related to the cessation of cell division. With the cessation of mitotic activity, the papilla cavity widens and then opens out completely. This is essentially an inversion of the epidermal bulb. The club consists of a hollow brush of keratinized cells at the end of the cortex. These cells would have become part of the compact cortex during the growth of the shaft. The inversion of the bulb leaves the basal layer at the apex of the papilla as a flat plate (cf. Schwanitz, 1938) or U-shaped layer of cells. In the ensuing hair generation, mitotic activity appears first in this germ. Mingled with the epidermal cells of the germ are the colorless, but potentially, melanogenic, cells, which are not easily detected. Taylor (1949) suggests that in the rat such cells are permanent and are merely reactivated to produce pigment with each hair generation. We confirm Taylor's observations that such cells remain colorless in albinos and are absent in piebald animals from follicles in white-spotted areas. With the inversion of the bulb, the papilla cells are held close to the germ, probably by shrinkage of the connective-tissue sheath of the follicle. It would seem that mitotic activity in the bulb produces a pressure which results in the compressed papilla and the shape of the bulb. When this pressure ceases at the end of the anagen, the bulb "relaxes." The apex of the basal layer within the bulb, on inversion, becomes the germ; the other matrix cells come to lie distally and in association with the loose keratinized cells of the club.

To return to the comparison with the skin, the nonmitotic papilla, like the skin dermis, is "nutritionally" important. The bulb is a hyperplastic Malpighian layer. The germ is a portion of the basal layer of the stratum Malpighii which remains dormant until the beginning of the next anagen.

SUMMARY

For experimental studies on the skin it is essential to understand in some detail the cyclic phenomena associated with the hair follicles. The anagen is divided into six substages, which are described along with the catagen and telogen in terms of hair development and pigmentation. Emphasis is placed on the basic cellular and developmental similarity of skin epidermis and hair

bulbs, i.e., Malpighian layers. The nature of the hair germ and the distribution and function of pigmentary dendritic cells are discussed.

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PLATE 1

All photomicrographs are of unstained material photographed with the oil-immersion, B minus, contrast-low objective of a Spencer phase-contrast microscope (except Fig. 2). The tissues were fixed in formol-calcium and sectioned by the freezing technique at approximately 5 μ. All are skin and hair sections of C57 Black mice.

FIG. 1.—Pigmentary dendritic cell in the basal layer of the epidermis of a 6-day-old mouse. The arrow indicates this cell at the dermal-epidermal junction. Note at the right a portion of a pigmented hair shaft. ×730.

FIG. 2.—Pigmentary dendritic cell, extending from skin epidermis into a newly invaginating follicle of a 3-day-old mouse. The arrow indicates this cell. Note at the lower left a pigmented hair shaft, the tip of which is at the stratum granulosum. This section has been treated with dopa to bring out the potential pigment cells and was photographed under regular (not phase) high-dry magnification. ×327.

FIG. 3.—Newly invaginating follicle of a 1-day-old mouse. No melanogenic pigment cells are visible. The layer of cells at the lower end is essentially the germ and will form the bulb. ×730.

FIG. 4.—Substage Anagen II, from skin plucked 2 days previously. The bulb is forming by downgrowth of germ cells around the papilla cells, and above the germ is the beginning of the inner-sheath dome. The outline of the papilla cavity and the position of the dome are indicated by the ink lines. Note the pigment remnant at the right edge of the bulb. ×730.

FIG. 5.—Pigmentary dendritic cell of substage Anagen III from a 2-day-old mouse. The focus is on the outside of the bulb. ×730.

FIG. 6.—Substage Anagen III from a 1-day-old mouse. Note the papilla cavity and the pigment-cell distribution of the bulb. ×730.

FIG. 7.—Substage Anagen III from skin plucked 4 days previously. The region is just distal to the main portion of the bulb. Note the elongating internal sheath inside the external sheath. Note that no hair shaft is present as yet. Some characteristic pigment remnants are present at the outer edge of the bulb and above and are not to be confused with the newly forming pigment. ×730.

PLATE 2

All photomicrographs are of unstained material photographed with the oil-immersion, B minus, contrast-low objective of a Spencer phase-contrast microscope. The tissues were fixed in formol-calcium and sectioned by the freezing technique at approximately 5 μ. All are skin and hair sections of C57 Black mice (except Fig. 11).

FIG. 8.—Substage Anagen IV from skin plucked 6 days previously. Certain upper matrix cells have now been "inoculated" with pigment granules. Note the shape of the papilla cavity and the cellular arrangement of the lower bulb. ×980.

FIG. 9.—Substage Anagen IV from a 6-day-old mouse. The focus is toward the outside of the bulb. Note the pigmentary dendritic cell, which is unusually low in the bulb and is therefore not masked by the dense pigmentation of the upper matrix cells. ×980.

FIG. 10.—Substage Anagen V from skin plucked 8 days previously. The compressed and frequently acentric papilla cavity characteristic of this substage is retouched with ink lines. At this stage the tip of the hair shaft is just reaching the surface, and the skin appears dark by gross observation. ×980.

FIG. 11.—Telogen stage. This resting stage was taken from the white area of a piebald mouse. The arrow indicates the germ plate (not always so biconcave as seen here). The club with its associated cells is seen above the germ; the ball of papilla cells is seen below the germ. ×980.

PLATE I

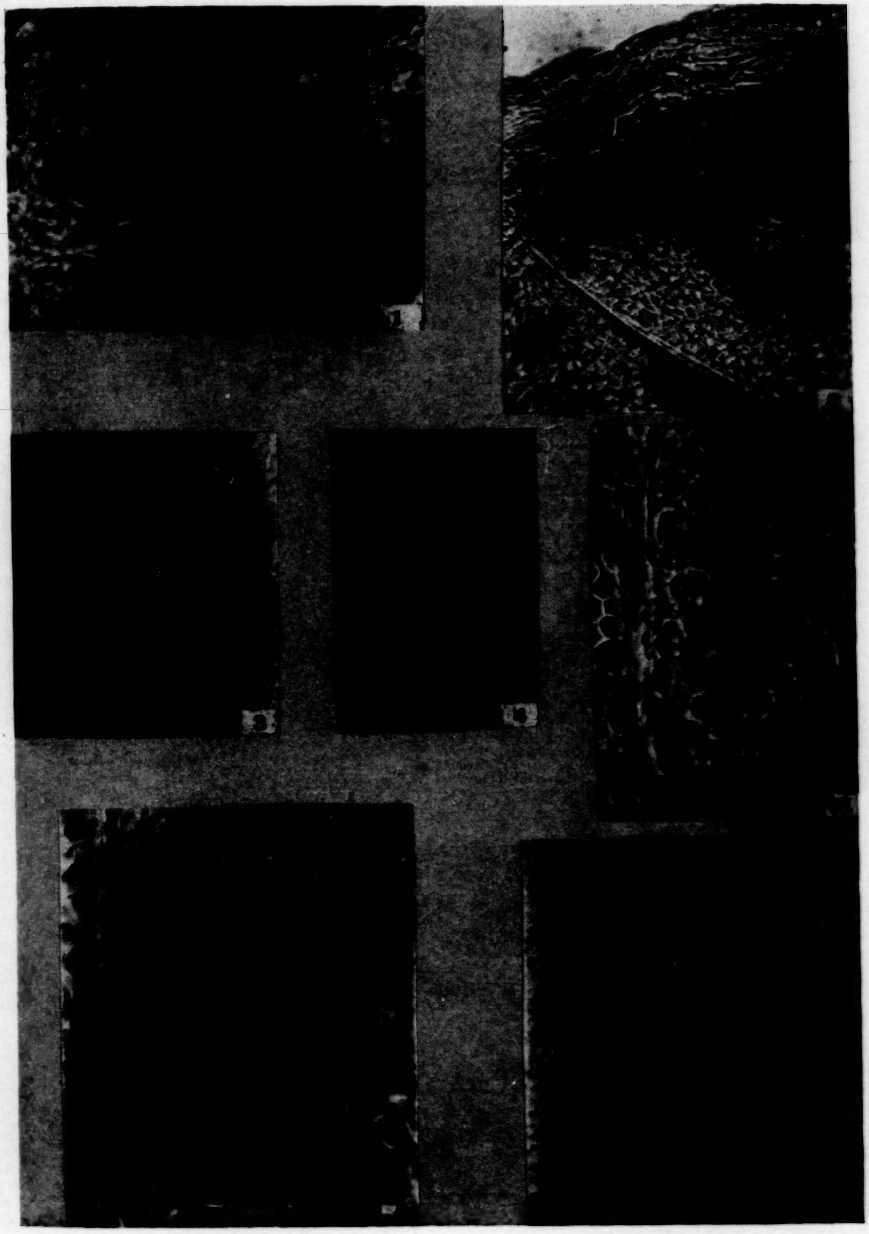
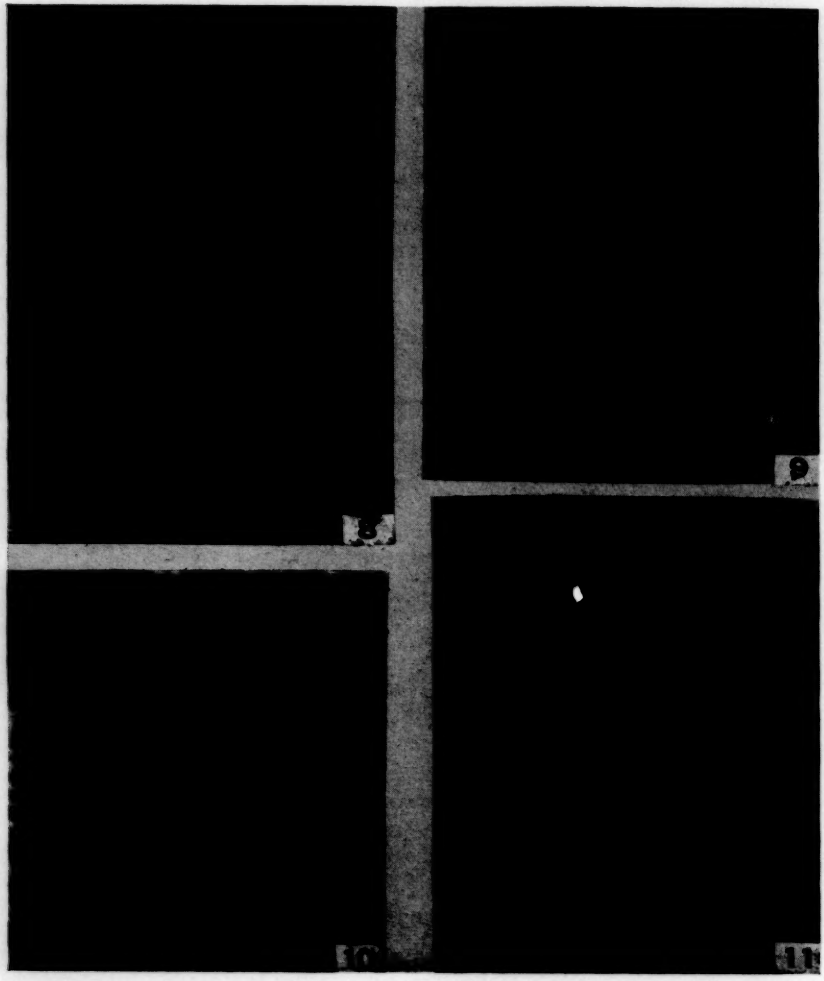


PLATE II



THE SEASONAL REPRODUCTIVE CONDITION OF FEMALE NORWAY (BROWN) RATS IN BALTIMORE, MARYLAND[1]

DAVID E. DAVIS AND OCTAVIA HALL

IN ORDER to understand the role of reproduction in population changes, a collection of Norway rats (*Rattus norvegicus*) was obtained in Baltimore, Maryland. An analysis of the reproductive condition of the males (Davis and Hall, 1948) showed that males produced sperm at all months of the year. Hence, if seasonal changes in pregnancy rates occur, they must be caused by changes in the female or by some changes in behavior patterns. This paper describes the reproductive condition of the females during all seasons of the year.

Since the paper on males (Davis and Hall, 1948) describes the methods in some detail, only a brief statement is necessary here. The rats were captured in poultry warehouses at regular intervals and brought to the laboratory alive for autopsy. For the females the following data were recorded: location of capture, date, weight, length of head plus body, third molar erupted or not, skull ossified or not, vagina perforate or imperforate, number of visible nipples, lactating or not, size of uterus (four grades), vascularity of uterus (four grades), number of embryos macroscopically visible, and number of placental scars. These data were coded and transferred to cards for mechanical sorting and tabulation. The entire reproductive tract was removed, fixed in Bouin's solution, and stored in alcohol. Later the embryos were recounted, resorbing embryos were recorded, and the length of the median embryo of each pregnant female was measured. The ovaries and frequently the oviducts (not uterus) were sectioned serially.

To divide the year into biologically meaningful parts, a hythergraph was made to show the weekly mean temperature and weekly precipitation from January 1, 1946, to March 3, 1947. By these data the year was divided into eight seasons (for details see Davis and Hall, 1948): *hibernal* (1946), January 1–February 25; *prevernal*, February 26–May 6; *vernal*, May 7–June 24; *estival*, June 25–August 19; *postestvial*, August 20–September 30; *serotinal*, October 1–November 11; *autumnal*, November 12–December 16; *hibernal* (1947), December 17–March 3.

COMPOSITION OF THE MATERIAL

The numbers of females according to size are tabulated in Table 1 and diagrammed in Figure 1. As might be expected, a large number of small (presumably young) rats was captured in the spring (prevernal and vernal seasons) and in the fall (postestival, serotinal, and autumnal seasons). The large number of small female rats in the first hibernal season is surprising. The explanation may involve some mortality factor rather than a seasonal reproductive phenomenon.

A comparison of weights of several populations may be obtained by the calculation of $K = W/L^3$ (W is weight and

[1] Contribution from the Rodent Ecology Project of the department of parasitology, School of Hygiene and Public Health, The Johns Hopkins University and the Carter Physiological Laboratory, University of Texas Medical Branch, Galveston, Texas. The work reported in this paper was conducted under a grant from the International Health Division of the Rockefeller Foundation.

L is length) for the rats for each season. When the value of K is high, the rats are relatively heavy in comparison to length. The values (Table 3) are rather constant, except for a strikingly low value in the estival season indicating relatively low weights in relation to length.

pregnancy rates, a study of the ovaries of 276 rats with perforated vaginas was made. These rats were a random sample of the population, since the only rats not studied were those for which the material was lost or inadequately preserved. The ovaries were sectioned serially and ex-

TABLE 1

WEIGHT COMPOSITION BY SEASONS

Season	Female Rats	Percentage in Weight Class (Gm.)					
		25–99	100–199	200–299	300–399	400–499	500–599
Hibernal	117	44	20	23	9	4	0
Prevernal	112	36	27	13	14	10	0
Vernal	57	37	14	28	19	2	0
Estival	112	18	21	20	29	12	0
Postestival	54	41	20	17	17	5	0
Serotinal	60	39	24	20	10	7	0
Autumnal	43	35	28	18	12	7	0
Hibernal	42	24	19	21	24	7	5

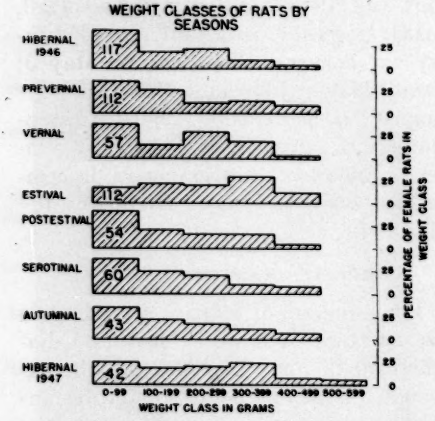


FIG. 1.—Percentage of females in weight classes in each season. The numbers represent the total females examined in each season.

The sex ratio in the collection of 1,318 rats was 54.4 per cent males, which differs significantly ($P < 0.005$) from 50 per cent males. No consistent seasonal changes in sex ratios were apparent.

SEASONAL REPRODUCTIVE CONDITION

PREGNANCY

a) Frequency of estrus.—In an attempt to understand the causes of variations in

TABLE 2

FREQUENCY OF GENERATIONS OF CORPORA LUTEA

Season	Rats Examined	Per Cent with	
		1 Generation	2 Generations
Hibernal (1946)	39	46	54
Prevernal	46	41	59
Vernal	27	48	52
Estival	75	37	63
Postestival	24	25	75
Serotinal	27	22	78
Autumnal	15	60	40
Hibernal (1947)	23	43	57
Total	276	.....	.....

amined for generations of corpora lutea (Hall and Davis, 1950). It is assumed that a rat showing only one generation of corpora has had less frequent estrous cycles than has a rat showing two generations. Table 2 shows the results of examination of 276 pairs of ovaries. Pregnancy should be more prevalent at times of frequent estrus. It is at once apparent that the frequency of two generations of corpora increased gradually until the serot-

inal season and then dropped in the autumnal season. If the assumptions are valid and if the data are representative, then we should expect a gradually increasing prevalence of pregnancy until the drop in the serotinal season. Analysis of these data by size of rat does not reveal any significant differences from season to season, although the data are not adequate when divided into categories.

b) Pregnancy rate.—The seasonal changes in reproductive condition culminate in changes in pregnancy rates. These, however, are not simple calculations, because changes in the age composition of the sample make it necessary first to define the populations being compared. Obviously, to include male rats or young female rats is absurd. Therefore, the universe sampled includes only adult female rats. "Adult" also requires definition to include only those rats which can be expected to be potential breeders. One definition could be based upon size by stating that a female more than 199 mm. in length is adult because in our sample 98 per cent of such females have a perforate vagina. Likewise, a criterion of weight could be used. Another definition could be that a female having 10–12 visible nipples is adult because 80 per cent of the rats with 10–12 nipples have conceived and the others presumably are able to conceive. Still another definition could be that rats with a perforate vagina are adult because copulation is possible, although ovulation has not necessarily begun.

The results of calculations of pregnancy rates by these definitions of maturity —length, nipple number, and perforate vagina—are shown in Table 3 and Figure 2. Seasonal changes are apparent in the figures for all rats with a perforate vagina and for rats that are less than 200 mm. long. However, for rats that are over 199 mm. long or that have 10–12 nipples the seasonal differences are not statistically significant. An analysis of the size composition of the rats at different seasons by comparing R (the ratio of mean weight of rats with 12 nipples to the mean weight of rats weighing more than 74 gm.) shows that, in the vernal and estival seasons and again in the serotinal season , the rats with 12 nipples are relatively small (Table 4). The figures for mean length of slightly smaller rats (10–12 nipples) show the same relation. This means that rats at these two periods of the year can reproduce at a smaller size. Changes in reproductive rates therefore depend upon the changes in the size at which rats reproduce. It is apparent that the large females are responsible for a large share of reproduction.

c) Litter rate.—The number of litters produced by a female may be calculated (Emlen and Davis, 1948). It is desirable to change the notations from that publication so that $I_P = Pt/18$, where I_P is the incidence of pregnancy during collecting time, t, in days and P is the prevalence of pregnancy in the sample. To place the figures on an annual basis, multiply by $365/t$, which gives $I_P = 20.3P$. The variability can be indicated by calculating the standard deviation ($\sqrt{pq/n}$) and indicating the litter-rate limits by $I_P = 20.3\ (P + 2\ \text{S.D.})$ and $I_P = 20.3\ (P - 2\ \text{S.D.})$ to give the range at a confidence level of 0.05.

For the rats from these poultry warehouses the litter rates may be computed (Table 5). In interpreting these calculated rates it is obvious that they are statistical averages and that they should be applied only to the sample from which they were drawn. Furthermore, these calculations, like all averages, "average out" such factors as size, differences, length of life (e.g., only rarely does a female live for a year), seasonal changes, etc. In spite of these variations, the rates can be used to indicate the average number of embryos produced during a year.

TABLE 3

PREVALENCE OF PREGNANCY AND LACTATION BY SEASONS

Category	Hibernal	Prevernal	Vernal	Estival	Postestival	Serotinal	Autumnal	Hibernal	Total
Rats more than 199 mm. long	25	35	19	61	18	13	8	17	196*
Per cent pregnant	*52.0*	*38.1*	*47.4*	*36.1*	*44.5*	*30.8*	*37.5*	*41.1*	*40.3*
Rats more than 199 mm. long	25	36	19	62	18	13	8	18	199*
Per cent lactating	*28.0*	*36.1*	*63.1*	*66.2*	*77.8*	*69.2*	*62.5*	*44.5*	*54.7*
Rats with 10–12 nipples	40	56	30	81	26	36	21	27	317*
Per cent pregnant	*40.0*	*30.4*	*43.3*	*30.8*	*34.6*	*41.7*	*13.6*	*25.9*	*32.9*
Rats with 10–12 nipples	28	53	30	82	26	37	21	28	305*
Per cent lactating	*39.2*	*26.4*	*56.5*	*52.4*	*65.2*	*48.7*	*42.8*	*35.7*	*45.5*
Rats with perforate vagina	85	80	38	93	39	41	28	33	437*
Per cent pregnant	*18.8*	*20.0*	*36.9*	*26.9*	*23.1*	*36.6*	*10.7*	*18.3*	*23.6*
Rats with perforate vagina	85	81	38	94	39	40	25	32	434*
Per cent lactating	*12.9*	*17.3*	*44.7*	*45.7*	*43.5*	*45.0*	*36.0*	*31.2*	*32.0*
Rats with perforate vagina but <200 mm. long	60	45	19	32	21	28	20	16	241*
Per cent pregnant	*5.0*	*6.7*	*26.3*	*9.4*	*4.8*	*39.3*	*0*	*0*	*10.8*
Rats with perforate vagina but <200 mm. long	60	45	19	32	21	27	17	14	235*
Per cent lactating	*6.7*	*2.2*	*26.4*	*6.3*	*14.3*	*33.3*	*21.0*	*11.8*	*12.8*
Total embryos	157	150	112	229	88	144	28	71	979
Embryos per pregnant rat	9.8	9.4	8.0	9.1	9.8	9.6	9.3	10.1	9.2
$K = W/L^3$ (multiplied by 10^5)	3.40	3.72	3.72	3.11	3.84	3.87	3.81	3.63	3.6
Rats with perforate vagina	85	80	38	93	39	39	25	31	430
Percent both pregnant and and lactating	*5.9*	*6.2*	*10.5*	*16.1*	*15.4*	*28.2*	*4.0*	*12.9*	*11.8*

* The size of samples for calculating pregnancy and lactation differ slightly because data were lacking for one or the other stat in a few cases.

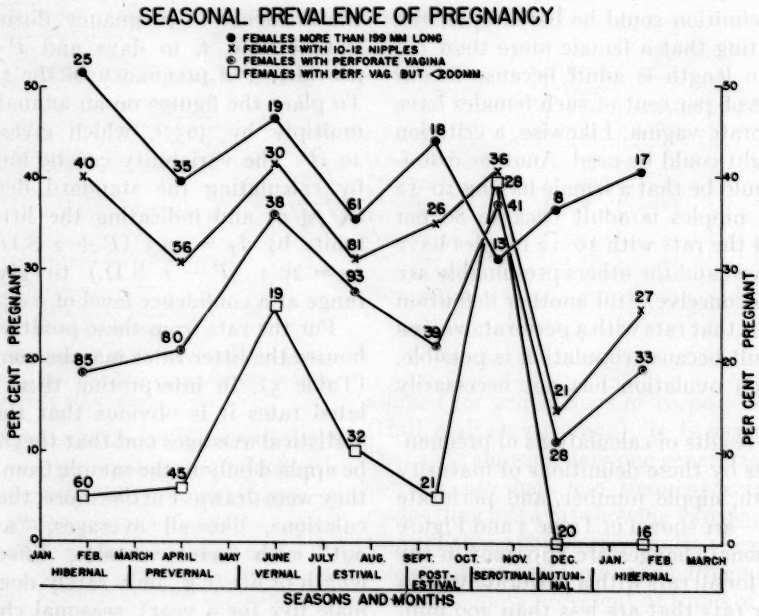

SEASONAL PREVALENCE OF PREGNANCY
FEMALES MORE THAN 199 MM LONG
FEMALES WITH 10-12 NIPPLES
FEMALES WITH PERFORATE VAGINA
FEMALES WITH PERF. VAG. BUT <200MM.
PER CENT PREGNANT
JAN. FEB. MARCH APRIL MAY JUNE JULY AUG. SEPT. OCT. NOV. DEC. JAN. FEB. MARCH
HIBERNAL PREVERNAL VERNAL ESTIVAL POST-ESTIVAL SEROTINAL AUTUM-NAL HIBERNAL
SEASONS AND MONTHS

FIG. 2.—Prevalence of pregnancy in each season. The numbers above each symbol refer to the number of rats examined.

d) Relation of pregnancy to weight.—An analysis of the relation between weight of rat and prevalence of pregnancy gave the regression equation $y = -25.4 + 0.186x$, where y is the prevalence of pregnancy and x is the weight in grams (Fig. 3). The weight of pregnant females was not corrected by subtracting the weight of the embryos, since their weight is significant only during the last few days of pregnancy. The increase in prevalence is significant ($P = 0.005$), accords with data from other places (Davis, 1947; Perry, 1945), and is in sharp contrast to the results of laboratory tests (King, 1916). Apparently, in nature increased size favors pregnancy. The data for the relation between length of rat and prevalence of pregnancy gave similar results.

e) Number of embryos.—The mean number of embryos in 105 pregnant females was 9.28 ± 0.28 (standard error). There is some increase in the number of embryos with increase in size of the rat. The correlation coefficient for embryos and length is +0.105, and the regression equation is $y = 6.32 + 0.0136x$, where y is the number of embryos and x is the length in millimeters. This correlation is not significant and could not result in significant changes in numbers of embryos due to size differences of the females that were captured at different seasons.

Seasonal differences in the number of embryos per pregnant female are rather

TABLE 4

SEASONAL DIFFERENCES IN SIZE IN RELATION TO NUMBER OF NIPPLES

Season	Rats with 10–12 Nipples			Rats with 12 Nipples			
	No.	Mean Length	S.D.	No.	Mean Weight	S.D.	Ratio*
Hibernal (1946)	27	211.7	17.9	16	351.5	100.0	1.61
Prevernal	43	205.5	26.8	39	333.7	83.8	1.43
Vernal	30	206.3	15.3	29	318.5	58.5	1.24
Estival	79	215.0	23.8	68	340.4	87.8	1.23
Postestival	23	205.9	15.2	19	346.7	74.2	1.53
Serotinal	32	193.4	23.5	30	300.0	102.0	1.24
Autumnal	21	195.5	25.2	18	313.5	99.9	1.47
Hibernal (1947)	27	208.0	25.8	26	340.3	111.2	1.34

* Ratio of mean weight of rats with 12 nipples to weight of all rats weighing more than 74 gm.

TABLE 5

INCIDENCE OF PREGNANCY AND OF LACTATION*

Category of Females	Pregnancy			Lactation			Diff. $I_P - I_L$	Per Cent Loss	Min. Per Cent Loss	Max. Per Cent Loss
	I_P	+2 S.D.	−2 S.D.	I_L	+2 S.D.	−2 S.D.				
Less than 200 mm. long	2.2	2.6	1.8	1.3	1.7	0.8	0.9	41.0	5.6	69.2
Perforate vagina	4.8	5.2	4.3	3.2	3.7	2.8	1.6	33.3	13.8	46.0
10–12 visible nipples	6.7	7.2	6.2	4.6	4.9	4.3	2.1	31.4	21.0	40.2
More than 199 mm. long	8.2	8.9	7.5	5.5	6.2	4.8	2.7	32.9	14.6	46.2

* I_P = Incidence of pregnancy; I_L = Incidence of lactation; +2 S.D. = Upper limit of incidence ($P = 0.05$); −2 S.D. = Lower limit of incidence ($P = 0.05$).

slight (Table 3) and do not indicate any seasonal trends, although more data might reveal such differences. The low number in the vernal season is not significantly different from the number in the estival season.

An examination for resorption of implanted embryos showed that 23 per cent of the pregnant females had at least 1 resorbing embryo and that there was a total of 67 resorbing embryos, or about 6 per cent of the total number of embryos.

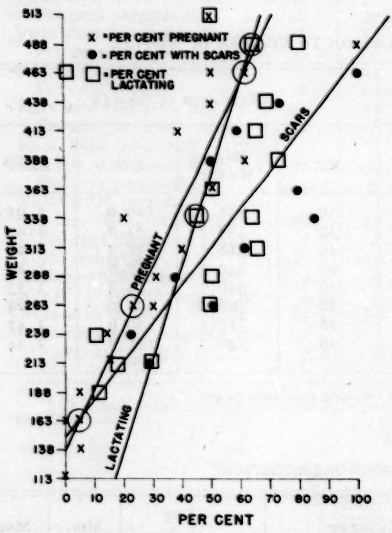


FIG. 3.—Relation between weight (gm.) and pregnancy, lactation, and presence of placental scars.

f) Embryo rate.—This statistic obviously is the litter rate multiplied by the number of embryos to give the number of embryos produced in a given time, e.g., in a year. The annual embryo rate for females with a perforate vagina is 4.79×9.28, or 44.4 embryos. For females with 10–12 visible nipples it is 6.68×9.08, or 66.9 embryos. For females more than 199 mm. long it is 8.20×9.62, or 78.9 embryos. These values emphasize the fact that the differences in litter rate are much more influential than differences in number of embryos per female in determining the reproductive rate.

LACTATION

The analysis of seasonal changes in prevalence of lactation also requires a careful definition of the population being examined. Table 3 and Figure 4 show the prevalence of lactation in rats more than 199 mm. long, in rats with 10–12 visible nipples, in all rats with a perforate vagina, and in rats with a perforate vagina but less than 200 mm. long. These prevalences differ strikingly from the prevalence of pregnancy in that there is an increase in the summer. The greater reproductive success of the larger females is apparent.

It is also possible to indicate the number of litters suckled, by calculations similar to those for incidence of pregnancy. The lactation period of wild rats is about 35 days (Donaldson, 1924), although the young can be weaned much earlier. Hence $I_L = Lt/35$, or, on a yearly basis, $I_L = 365L/35 = 10.5L$ (where I_L is incidence of lactation and L is proportation of adult females that were lactating during time t). Since the 35-day period is an approximation, the formula can be written simply $I_L = 10L$. Obviously, the confidence limits can be calculated by the same method as that used for litter rates. Table 5 gives the results of these calculations.

It must be noted that these calculations include the loss of suckling rats during lactation. Under natural conditions some loss of entire litters occurs, thereby, in effect, lowering the average length of lactation to an unknown extent. The extent of this loss during suckling may be estimated by determining the ratio of prevalence of pregnancy to prevalence of lactation. If there is no loss during the suckling period, then the

ratio should be 18/36 = 0.5. Actually, from Table 3, the ratios are higher: for rats more than 199 mm. long, 0.735; for rats with 10–12 nipples, 0.728; for rats with perforate vagina, 0.737; for rats with perforate vagina but less than 200 mm. long, 0.842. Averaging the first three ratios gives 0.733. Then, calculating the average duration of lactation, $D = 18/0.733 = 25$ days.

The ratio of prevalence of pregnancy to prevalence of lactation for rats with a perforate vagina but less than 200 mm. long is high (0.842), thereby giving the mean duration of lactation as 21 days. The survival of complete litters during lactation may be calculated from the ratio of average duration to normal duration, or $21/35 \times 100 = 60$ per cent for these smallest rats. For the other rats, $25/35 \times 100 = 71$ per cent survival during lactation. These very small rats, therefore, have a low survival of suckling young.

An alternative method for estimating the loss is to compare directly the incidence of pregnancy with the incidence of lactation. A comparison of the categories of females (Table 5) shows losses ranging from 24 to 41 per cent, which agrees with the results for survival as calculated in the method above. It may be noted that the limits (confidence level of $P = 0.05$) do not overlap in any category. The minimum loss can be estimated by comparing the lower limit of incidence of pregnancy with the upper limit of incidence of lactation (Table 5). Similarly, the maximum loss can be estimated by comparing the upper limit of the incidence of pregnancy with the lower limit of the incidence of lactation (Table 5).

The number of lactating females increases with size. The regression equation

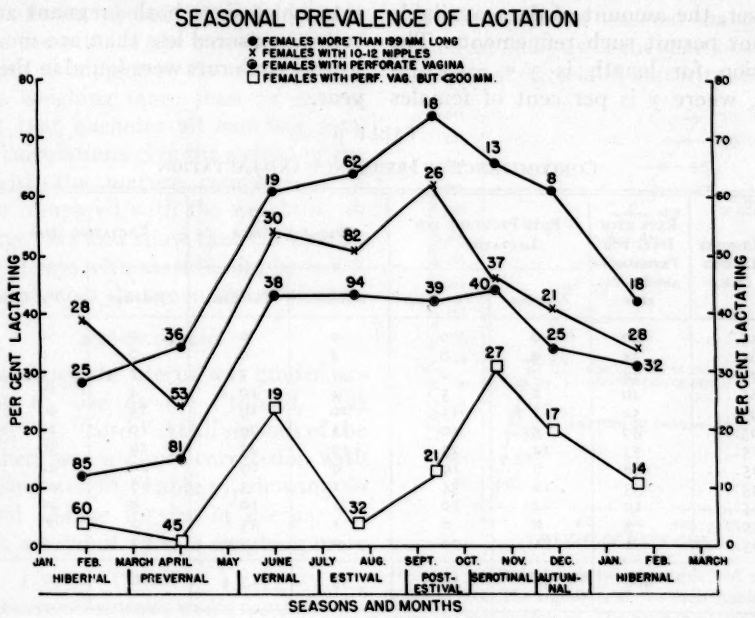

SEASONAL PREVALENCE OF LACTATION
● FEMALES MORE THAN 199 MM. LONG
X FEMALES WITH 10-12 NIPPLES
◉ FEMALES WITH PERFORATE VAGINA
□ FEMALES WITH PERF. VAG. BUT <200 MM.
PER CENT LACTATING
JAN. FEB. MARCH APRIL MAY JUNE JULY AUG. SEPT. OCT. NOV. DEC. JAN. FEB. MARCH
HIBERNAL PREVERNAL VERNAL ESTIVAL POST-ESTIVAL SEROTINAL AUTUM-NAL HIBERNAL
SEASONS AND MONTHS

FIG. 4.—Prevalence of lactation in each season. The numbers above each symbol refer to the number of rats examined.

for weight (Fig. 3) is $y = 3.22 + 0.124x$, where y is per cent of females which are lactating and x is weight in grams. It is possible that the true relation is a curve (concave to the left) that reaches a maximum at about 60 per cent lactating, as indicated by the squares in Figure 3. However, the amount of data available does not permit such refinements. The regression for length is $y = -57.6 + 0.473x$, where y is per cent of females which are lactating and x is length in millimeters.

phasize the preponderance of reproduction by large rats.

Seasonal changes in concomitance of pregnancy and lactation (Table 3) show a steady increase during the summer months. The maximum in the serotinal season is noteworthy because 7 of the 11 rats which were both pregnant and lactating measured less than 200 mm. Only 3 other such rats were found in the entire year.

TABLE 6

CONCOMITANCE OF PREGNANCY AND LACTATION

Midpoint Length (Mm.)	Rats with Data for Pregnancy and Lactation	Both Pregnant and Lactating		Pregnant Only		Lactating Only	
		Number	Per Cent	Number	Per Cent	Number	Per Cent
155.....	40	0	0	0	0	0	0
165.....	34	0	0	2	6	0	0
175.....	44	1	2	1	2	2	4
185.....	40	2	5	4	10	4	10
195.....	52	7	13	10	19	15	29
205.....	49	5	10	14	29	10	20
215.....	53	12	23	12	23	16	30
225.....	48	9	19	9	19	20	42
235.....	35	12	34	1	3	16	46
245.....	10	2	20	1	10	4	40
255.....	3	0	0	1	33	2	66
265.....	1	1	100	0	0	0	0
	409	51	12.5	55	13.4	88	21.5

CONCOMITANCE OF PREGNANCY AND LACTATION

The frequency of litters may also be examined from the simultaneous occurrence of pregnancy and lactation. Rats that are both pregnant and lactating presumably became pregnant at the *post partum* estrous period. From Table 6, of the 409 rats for which data were available for both pregnancy and lactation, only 12.5 per cent were both pregnant and lactating. The data do not show any striking differences according to size in the three categories of rats, except to em-

PERFORATION OF THE VAGINA

In microtine and some other rodents the vagina closes in the nonbreeding season and hence indicates lack of activity. In the Norway rats of this collection, since no large rat (over 124 gm. or 190 mm.) had an imperforate vagina, it is concluded that there is no seasonal closure of the vagina. Furthermore, no seasonal differences in the size of the rat at perforation of the vagina were apparent from these data.

NUMBER OF NIPPLES

At autopsy the number of nipples readily visible by blowing aside the hair was recorded. Table 4 shows the mean

weight of the rats with 12 visible nipples and the mean length of rats with 10–12 nipples. It is apparent that in certain seasons (prevernal, vernal, serotinal, and autumnal) the rats with the mature complement of nipples average smaller than at other seasons.

The column labeled "Ratio" in Table 4 gives the ratio of the mean weight of rats with 12 nipples to the mean weight of rats weighing more than 74 gm., a weight that excludes all suckling rats. These calculations give the weight of the rats with the mature complement of nipples compared with the weight of all the large rats and show that the change in size of rats with mature nipples is not due merely to a change in size of all rats.

SIZE OF UTERUS

At autopsy the uterus was graded according to size as (1) "thread," (2) "string," (3) "cord." At all seasons of the year there was a direct correlation with increasing weight (Table 7), showing no seasonal change in size of uterus. Although a total of 17 rats weighing more than 300 gm. had either a thread or a string uterus, these rats were collected in various seasons and hence do not represent a seasonal regression in the size of the uterus such as occurs, for example, in microtines (Hamilton, 1937). The size of the uterus has no value as a criterion of maturity because thread-sized uteri can be found in 6.6 per cent of rats that have placental scars. Thus, from the size of the uterus it is not possible to know whether the rat has produced young or not.

TABLE 7

RELATION BETWEEN WEIGHT OF RAT AND SIZE OF UTERUS

Weight (Gm.)	Thread	String	Cord	Total
0–99	188	6	0	194
100–199	63	44	17	124
200–299	8	27	77	112
300–399	4	8	87	99
400–499	0	5	25	30
500 and over	0	0	2	2
Total	263	90	208	561

CRITERIA OF MATURITY

These studies of seasonal changes permitted an analysis of the relations of size and several criteria of maturity. Maturity, of course, can be defined in a number of ways, depending upon the purpose of the investigation. The size (weight or length) at which each criterion is attained can then be determined. Before defining the criteria and determining their relation to size, it is desirable to describe the relation of weight and length in the females of this population.

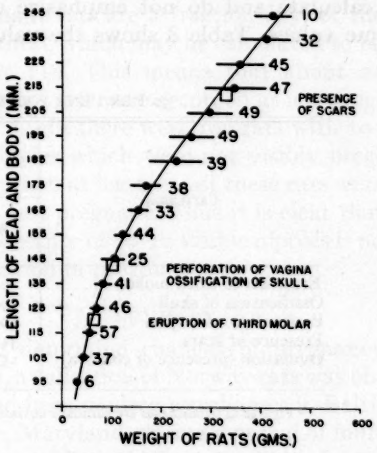


FIG. 5.—Relation between length and weight of female rats. The numbers above each symbol represent the rats examined. The horizontal line indicates 1 standard deviation on each side.

In Figure 5 the relations of weight and length are graphed. The upper portion of the curve is unusually straight, in contrast to the curve for males (Davis and Hall, 1948). A detailed analysis of the data gave no indication of an explanation. Apparently, female rats continue to

increase in weight as long as they increase in length, within the range of lengths and weights here obtained. Possibly at higher weights the curve would become concave.

MORPHOLOGICAL CHARACTERS

For several characters a useful measure is the size (weight or length) at which 50 per cent of the rats possess the character. These 50 per cent points are simple to calculate and do not emphasize extreme values. Table 8 shows the values for the eruption of the third molar tooth, for the ossification of the skull, for the perforation of the vagina, for ovulation, and for the presence of placental scars. These five characters can be used to compare relative ages of rats at different parts of the weight range. For example, eruption of the third molar is useful for small rats and the presence of placental scars for large rats. Thus, if a rat weighing 50 gm. had a perforate vagina, we would be fairly confident that it was fairly old and had lost weight for some reason, because it should have weighed about 100 gm. Another use of these criteria is to compare growth rates at different localities. If the ratios of 50 per cent point/mean weight are significantly different, then it can be concluded that growth rates differ.

TABLE 8

50 PER CENT POINTS FOR MATURITY CRITERIA*

Criterion	50 Per Cent Point		Ratio of 50 Per Cent Point to Mean	
	Weight (Gm.)	Length (Mm.)	Weight (Gm.)	Length (Mm.)
Eruption of third molar	64(35)	119(15)	0.33	0.71
Ossification of skull	98(45)	143(56)	0.51	0.85
Perforation of vagina	102(77)	143(39)	0.53	0.85
Presence of scars	318(272)	216(90)	1.64	1.28
Ovulation (presence of corpora)	153(34)	144(26)	0.79	0.86

* Figures in parentheses are standard deviations.

The presence of scars in the uterus is good indication that implantation has occurred. An analysis of the presence or absence of scars in relation to size of rat showed a correlation coefficient between number of scars and length of rat of only +0.147, which is not significantly different from zero. While it is known that the number of scars is only a rough indication of the number of young produced (Davis and Emlen, 1948), this lack of correlation suggests that size of female (age) has little relation to the number of implantations.

PRESENCE OF NIPPLES

In an attempt to find external characters which would, without injury, provide information about the reproductive history and maturity of the female, the relations between the number of visible nipples and a number of reproductive characters were examined. The number of nipples could be seen by blowing the hair on the ventral surface. The maximum is six in the pelvic and six in the pectoral region.

a) Weight and length of rat.—As would be expected, a correlation between weight and number of nipples exists (Table 9). Because of the considerable spread and overlap of the distributions, the relation-

ship has little predictive value. Hence, no correlation coefficients or means were calculated. The relation of number of nipples and length is very similar to that of weight.

b) Perforation of the vagina.—Of the 190 rats with an imperforate vagina, only 12 had visible nipples, mostly less than 6. Of the 415 rats with a perforate vagina,

TABLE 9

RELATION OF NUMBER OF NIPPLES AND WEIGHT

Midpoint of Weight Class (Gm.)	Visible Nipples			
	0	1–6	7–11	12
12.5	2	.....	.....	.....
37.5	59	.....	.....	.....
62.5	93	5	.....	.....
87.5	38	4	1	.....
112.5	23	5	4	3
137.5	15	9	10	4
162.5	9	8	6	13
187.5	.....	6	4	11
212.5	2	2	7	9
237.5	2	.....	6	24
262.5	.....	2	2	33
287.5	.....	.....	5	23
312.5	.....	.....	1	19
337.5	.....	1	1	22
362.5	.....	.....	4	26
387.5	.....	.....	3	26
412.5	.....	.....	3	18
437.5	.....	.....	.....	12
462.5	.....	.....	.....	2
487.5	.....	.....	.....	7
512.5	.....	.....	.....	2

78 per cent had 6 or more visible nipples. It seems clear that the nipples develop later than perforation of the vagina.

c) Placental scars.—A comparison of the number of nipples and the presence of scars showed that, of 382 rats without scars, 21 per cent had 10–12 nipples. Hence, the presence of 10–12 nipples is not proof that implantation has occurred. But of the 109 rats with scars, only 2 lacked nipples and only 4 had less than 10 nipples. This relationship shows that, if a rat has scars, it almost always has nipples, but this is of no use as an external indication of past reproductive history.

d) Pregnancy.—All of 96 visibly pregnant rats had nipples, and only 4 had less than 10 nipples. It is thus clear that the nipples develop when the rat becomes pregnant. The converse relation may be analyzed as follows: Since a rat is visibly pregnant for only 18 of the 22 days of pregnancy, some pregnant rats were recorded as not pregnant. The 96 pregnant rats are a fraction ($\frac{18}{22}$) of the real total, which may be calculated to be about 118. This means that about 22 pregnant rats were recorded as not pregnant. Since there were 196 rats with 10–12 nipples which were not visibly pregnant, then at least 174 of these rats were truly not pregnant. Thus it is clear that the presence of 10–12 visible nipples is no indication of pregnancy.

SUMMARY

To determine changes in pregnancy rates, a collection of Norway rats was obtained from poultry warehouses in Baltimore, Maryland, during a period of fourteen months, which were divided into eight seasons. Data on size and reproductive condition were obtained at autopsy.

The sample of 597 females contained a high proportion of small rats in the spring and fall and also at the beginning of collection. Of the 1,318 rats captured, 54.4 per cent were males.

The analysis of the prevalence of pregnancy showed no significant variation among seasons for the large rats and an average of 40.3 per cent pregnant. The prevalence for small rats was high in the spring and fall and low in the summer and winter and averaged 10.8 per cent. Annual litter rates varied from 8.20 for large rats to 2.19 for small rats. The mean number of embryos in 105 pregnant rats was 9.28 ± 2.28.

Seasonal changes in lactation were an

increase to a steady level for spring and summer and a sharp decline in the fall.

There is no seasonal closure of the vagina or regression of the uterus.

To determine relative ages of rats, the size at which 50 per cent of the rats have the third molar erupted (64 gm.) or the skull ossified (98 gm.) or the vagina perforated (102 gm.) or follicles ovulated (153 gm.) or placental scars present (318 gm.) may be calculated.

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ONTOGENETIC CHANGE IN THE ANTIGENIC SPECIFICITY OF THE CHICK SPLEEN[1]

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WEISS (1947) and Tyler (1947) have focused attention upon the fundamental problem of specificity in the growth of cellular constituents and have pointed out that the mechanism of the process of growth may be viewed as essentially analogous to the mechanism of antibody formation, in which antibody globulin of a specific structural configuration is produced in response to the incorporation of antigen in the site of synthesis. Whether this is accomplished by direct physical contact with the antigen template and consequent impression of a spatial configuration upon the gamma-globulin (hypothesis of Haurowitz and Mudd; further elaborated by Pauling, 1940) or whether it is accomplished by the formation of an adaptive enzyme capable of self-replication, resulting in the appearance of circulating antibodies (Burnet, 1941, 1948), is not a primary consideration, for either theory meets the demands of the autoantibody concept. The view of Haurowitz, Mudd, and Pauling, although it is widely accepted, has the disadvantage of limiting the production of antibody to that period in which antigen is present in the site of antibody production. In normal conditions of growth the constituents synthesized in a cell would be complementary to molecular configurations at the site of synthesis. Under certain conditions, organ antigen introduced into the cellular environment at a time of active synthesis might serve as an acces-

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sory template and thus catalyze the formation of additional homologous antigen. This would lead to the enlargement of the homologous organ.

In the first direct test of this hypothesis, Weiss and Wang (1941; also Weiss, 1947) reported that the incorporation of liver fragments from 6-day-old chick embryos into the area vasculosa of 4-day-old chick hosts elicited a striking response in the growth of the hosts' livers. After an initial decline, the weights of the hosts' livers steadily increased in comparison with controls until, on the seventh postoperative day, livers of experimental hosts were over 40 per cent heavier than those of controls. Histological studies revealed that the observed enlargement was reflected both in cellular hypertrophy and in increased mitotic rate. The effect, however, was not completely specific; enlarged livers were accompanied by somewhat enlarged kidneys; this correlated effect might be explained as an accumulation of toxic substances by the kidney. On the other hand, grafts of blood clots, skin, and mesonephros elicited some increase in liver growth.

There is no doubt that substances released from the grafts and carried into the embryo by the blood stream call forth an increase in the growth of the homologous organ of the host. Weiss has advanced, as a tentative hypothesis, that "either specifically shaped parts of liver cell proteins can act directly as nuclei for further synthesis when entering the appropriate environment, i.e., another liver cell, or they act merely as models for moulds which would turn out more of the original product." Unquestionably, this interpretation is in accord with the evidence presented; furthermore, the results are in agreement with immunological studies on organ-specificity, which clearly show that each organ is actually composed of a complex of antigens, certain of these being organ-specific and others being held in common with one or more other organs (Landsteiner, 1945; Maculla, 1948*a*, *c*). Hence the nonspecific effects obtained by grafts of other tissues might be expected, since each graft contains a multiplicity of antigenic substances. In essence, then, this hypothesis postulates a quantitative difference in the amounts of specific antigenic substances in various organs. Liver cells, because they contain the highest proportion of specific liver antigens, would therefore have their primary effect on liver, and so on.

The desirability of extending this line of research is apparent. Among the pertinent questions it raises are the following: Since the effect is not completely organ-specific, can the relative effectiveness of organ grafts capable of evoking a response in the growth of a given organ be correlated with quantitative differences in organ antigens? Can a similar homologous response be obtained with other organ grafts? Is the stimulus class-specific, i.e., is homologous organ growth obtained only with organ grafts from donors of the same class?

In this regard it is somewhat surprising that so little attention has been directed to the remarkably similar enlargement in the homologous host organ following grafts of adult chicken spleen. The observation that chorioallantoic grafts of adult chicken spleen elicit a striking enlargement of the spleens of host chicks was first made by Murphy (1916) and was subsequently confirmed by Danchakoff (1916, 1918), Willier (1924, and unpublished results), and Pomerat (1949). Although considerable evidence has been presented bearing on the cellular nature of the response, the available data regarding the specificity and nature of the factors concerned in

evoking an enlargement of the host spleen following organ grafts are too contradictory and incomplete to permit any valid conclusion. Moreover, in spite of the current interest in the ontogeny of organ-specific proteins, the most challenging statement made by Danchakoff (1918), namely, that grafts of embryonic chick spleen evoke no response, has received scant attention. In fact, no data have been published to indicate the developmental periods at which the inability of grafts of embryonic spleen to elicit an enlargement of the host's spleen was determined. A confirmation of this provocative statement and a determination of the time at which embryonic tissues first become effective in stimulating enlargement of the host's spleen, combined with an immunological analysis of the ontogeny of specific spleen antigens, should afford an excellent opportunity to correlate chemical ontogeny with the onset of a specific physiological activity of engrafted spleen, viz., its ability to produce an enlargement of the homologous organ.

The purpose of the present investigation has been threefold: (1) to determine the class-specificity and the relative organ-specificity of those factors concerned in the enlargement of embryonic chick spleen following grafts of adult tissues; (2) to analyze experimentally the relative effectiveness of embryonic and adult spleen grafts in evoking a response of the host's spleen and to ascertain the time during development at which this capacity is first expressed; and (3) to attempt to correlate ontogenetic changes, measured by their physiological effects on the host organ, with changes detectable by immunological techniques, as a first step in an analysis of the precise nature of the substances involved.

OPERATIVE PROCEDURES AND RESULTS

In the analysis of the specificity and time of origin of the factors concerned in the enlargement of the embryonic chick spleen following homologous organ grafts, the initial approach has been to compare the effects on host embryo spleens of chorioallantoic transplants of the following: (1) adult chicken spleen, heart, and gonad, from several common breeds of fowl; (2) spleen from hatched chicks (1–49 days posthatching); (3) embryonic chick spleen from White Leghorn embryos of from 9 to 18 days' incubation; and (4) adult spleen from strain C57 mice, of both sexes. The technique of operation was essentially similar to that reported by Willier (1924, pp. 68–70). All transplants were made to the vascular chorioallantoic membrane of White Leghorn chick embryos incubated for 9 days at 37°.5–38°.0 C. In so far as possible, all transplants were of uniform size, 2 × 2 × 1 mm., except for spleen grafts from 9- and 12-day-old embryonic donors, in which cases either one or two entire spleens were transplanted. The grafts were permitted to grow for 8 days (total host age = 17 days), at which time they were recovered and fixed in Allen's B-15 or in Zenker-formol. The host embryos were stripped of all embryonic membranes, drained for 30 seconds on twenty sheets of No. 1 Whatman filter paper, and rapidly weighed to the nearest tenth of a gram on a triple-beam balance. Next, the host embryo spleens were removed, fixed for 6 hours in Bouin's picro-formol, and washed in five changes of 70 per cent ethyl alcohol. Each spleen was carefully trimmed, drained for 30 seconds on ten sheets of filter paper, and weighed to the nearest tenth of a milligram on a Roller-Smith precision balance. Other representative host spleens were preserved, for histological study only, in Allen's B-15 or

in Zenker-formol. Both grafts and host spleens were imbedded in paraffin, sectioned at 6 μ, and stained with either Heidenhain's iron haematoxylin or Delafield's haematoxylin-eosin azure.

Transplants were made to the chorioallantoic membranes of 669 host chicks; 243 well-incorporated grafts were recovered. In 61 additional saline controls the usual operative procedure was carried out, but only a drop of sterile Ringer-Locke solution was added; of the latter group, 56 survived until the seventeenth day of incubation, at which time their spleens were fixed for further study. The distribution of the transplants into the various classes and the incidence of successful grafts per group are shown in Table 1. The following points are noteworthy: (1) as might be expected, the grafts of embryonic chick spleen were incorporated most readily, 67 per cent being recovered as well-vascularized grafts; (2) there were no essential differences in the abilities of adult and recently hatched chicken spleen and of adult mouse spleen to survive on the chorioallantois; 30–40 per cent of these tissue transplants were successful.

In addition to the 56 saline controls recovered, 40 control grafts were examined. In this series various adult chicken tissues were transplanted. The following grafts were recovered: heart, 15; gonad, 15 (testis, 12; ovary, 3); pancreas, 4; brain, 3; liver, 3. The types of tissue transplanted as controls were selected in order to supplement the evidence previously presented by Danchakoff (1918) and Willier (1924). It appears well established that grafts of adult chicken liver and thymus (and possibly thyroid) are capable of evoking host spleen enlargement to a marked degree. In the present study, therefore, control grafts were made in order to determine the scope of the factors responsible. Since it appears to be essential for substances from the graft to be introduced into the circulation of the host, only those grafts which appeared to be well incorporated and supplied with branches of the umbilical blood vessels are included in the tabulation of data.

TABLE 1

WEIGHTS OF SPLEENS OF EMBRYONIC HOSTS FOLLOWING CHORIOALLANTOIC TRANSPLANTATION OF VARIOUSLY AGED CHICK AND ADULT MOUSE TISSUES

Donor Tissue	No. of Operations	Grafts Recovered	Per Cent of Successful Cases	Mean Weight of Host Spleens ± Standard Error of Mean (Mg.)	Standard Deviation
None	61	None (56 embryos)	91	5.3±0.19	1.4
Adult fowl heart, etc.	99	40	40	5.4±0.21	1.3
Adult mouse spleen	265	85	32	5.1±0.17	1.6
9–19-day embryo chick spleen	60	40	67	6.4±0.32	2.0
1–49-day hatched chick spleen	125	42	33	8.5±0.52	3.4
Adult fowl spleen	120	36	30	11.0±0.93	5.6

Grafts of embryonic spleen proved to be by far the most successful group not only in the percentage of grafts recovered but in ultimate graft size. Each of the 40 grafts of embryonic spleen recovered was considerably larger than at the time of its transplantation. In this group 31 grafts measured at least 3 × 3 × 2 mm., with the largest being 6 × 6 × 4 mm. On the other hand, in many cases it was difficult

to determine by gross observations whether grafts of adult chicken or mouse spleen or of hatched chick spleen had increased in size or had merely been maintained in the state at the time of transplantation. Histologically, however, engrafted adult spleen from both chicken and mouse was seen to contain apparently healthy splenic tissue in varying amounts. Mitotic figures were observed in several grafts. Quite often the side of the graft most removed from the vascular connections was necrotic, while the area nearest the vascular supply was growing. Lymphocytic and connective-tissue invasions of grafts were rarely observed. A more consistent reaction, however, was the presence of granulocytes, in considerable numbers, in grafts of adult or hatched chicken spleen or of adult mouse spleen. One point should be emphasized in this regard, namely, that there was little or no difference in the amount of granulocytic invasion in grafts from adult mouse or from adult or newly hatched chicken donors.

FACTORS CONCERNED IN THE ENLARGEMENT OF SPLEENS OF HOST EMBRYOS FOLLOWING GRAFTS OF ADULT TISSUES

Table 1 shows the mean weights of spleens in controls and in the several groups of experimental embryos. The mean weight of the spleens of 56 embryos (incubated 17 days) which received only a drop of saline was 5.3 ± 0.19 mg. The average spleen weight of the control group bearing grafts of adult chicken heart, gonad, etc. (40 cases), was 5.4 ± 0.21 mg. The difference between the means of the saline and the graft control groups is clearly not significant. For purposes of comparison with the average spleen weights of embryos bearing spleen grafts, therefore, the two control classes have been considered together, the average spleen weight of 96 controls being 5.3 ± 0.13 mg. In striking contrast, the average spleen weight of 36 embryos bearing grafts of adult chicken spleen was 11.0 ± 0.93, an increase of over 100 per cent. The difference between the mean weights in the two groups is highly significant, i.e. (Difference/S.E. of difference) = 6.1. These data indicate that the factors responsible for enlargement of the host spleen are peculiar to adult chicken spleen, being absent from chicken heart and gonad and probably from brain and pancreas. However, when these results are fitted into the over-all picture, i.e., are compiled with the results obtained by previous investigators, it is clear that the stimulus is only partially organ-specific; for, while homologous grafts evoke the greatest response, both liver and thymus are also effective. Although additional experiments have been conducted in an effort to evaluate the relative effectiveness of spleen, thymus, and liver, the results are too incomplete to justify any conclusion at this time. Consequently, they will be reported, together with the results of experiments now in progress, at a later date.

While the factors responsible for host spleen enlargement are only partially organ-specific, the results obtained in the present experiments indicate that the class-specificity of the stimulus is complete, i.e., that embryonic host spleens respond only to grafts of spleen from donors of the same class. The average spleen weight of 85 embryos bearing grafts of adult mouse spleen was 5.1 ± 0.17 mg., as compared with the control mean of 5.3 ± 0.13 mg. The difference between these means is clearly not significant, i.e., (Difference/S.E. of difference) = 1.

It is of considerable importance to note, at this point, that no significant differences were observed in over-all body

weights of host embryos following any of the types of grafts made. In general, a slight depression in body weight was found following chorioallantoic grafts, regardless of the donor tissue employed. Mean body weights of hosts bearing grafts were 6–10 per cent lower than the mean body weight of the saline control group. From these considerations and in view of the marked differences obtained, the organ weights have been compared directly rather than in terms of organ to body-weight ratios. Moreover, other lines of evidence indicate that spleen weights, at least in adult fowl, are independent of body weight and the weights of other organs (Oakberg and Lucas, 1949).

Histological studies, in which the enlarged spleens of chicks which had received grafts of adult chicken spleen were compared with control spleens and with spleens of chick embryos bearing grafts of adult mouse spleen, indicate the primary cause of the enlargement to be a stimulation of granulopoiesis. Intense proliferations of granulocytes have been found in all enlarged spleens examined to date. Although this analysis is still in progress, there have been no indications of any stimulation of erythropoiesis or of lymphopoiesis. Moreover, there are no unusual accumulations of erythrocytes undergoing destruction. These findings agree only in part with the results of previous analyses.

According to Murphy (1916), mammalian tumor fragments incorporated into the chick chorioallantoic membrane grow rapidly until the eighteenth or nineteenth day of incubation, at which time the host's immune mechanisms develop and destroy the graft. A number of subsequent investigations, however, have pointed to the fact that the chorioallantoic membrane normally loses its vascularity at this time, preparatory to hatching, and that tumor retrogression is the result of this natural change at the graft site. Murphy further stated that if adult chicken spleen grafts are made concomitantly with tumor grafts, the tumor tissue regresses at a significantly earlier stage. He attributed this effect to an induction of precocious species-specificity in the host and stated, moreover, that host spleen enlargement is due to a proliferation of small lymphocytes. This view has been challenged by Stevenson (1917), who repeated Murphy's experiments, using concomitant grafts of mammalian tumor and adult chicken spleen, and by Sandstrom (1932), who grafted adult chicken spleen in conjunction with duck kidney. In these investigations the heteroplastic transplants were unaffected by the presence of adult chicken spleen, despite the fact that the size of the host's spleen was markedly increased.

In Danchakoff's investigation (1916, 1918), the stimulatory action of adult spleen grafts on embryonic spleen served as a convenient tool in her analysis of the origin of blood cells. Her primary interest was in the nature of the response rather than in the mechanism of action of the stimulus. Histological studies of embryonic chick spleens stimulated by adult spleen grafts on the chorioallantoic membrane indicated that host spleen response was due to an intense stimulation and proliferation of mesenchyme and blood stem cells; moreover, the exact nature of the response varied according to the time at which the grafts were introduced. Since the stimulus had been the same in each case, host spleen response was considered to be a function of its developmental stage at the time of stimulation. According to Danchakoff, the chick spleen, which makes its appearance late in the fourth day of incubation, normally passes through three stages in its development: (1) The loose mesenchyme of

the primordium becomes more dense, and granulopoiesis begins. Grafts made in this period, i.e., during days 6–8, induce enlarged spleens which contain intense proliferations of granulocytes. (2) A stage of granulopoiesis and potential erythropoiesis (the latter not being realized normally to any significant extent) sets in. Grafts made during days 9 and 10 elicit, in some cases, intense erythropoiesis in host spleens, in addition to increased granulopoiesis, resulting in spleen enlargement. (3) The development of small lymphocytes, beginning at about the fourteenth day of incubation, characterizes the final period.

The data presented above are not compatible with the hypothesis advanced by Murphy. It appears clearly established that there is no appreciable proliferation or aggregation of lymphocytes in either the host spleen or the grafts. With one exception, however, Danchakoff's observations have been corroborated. In the present study no intense erythropoiesis has been observed concomitant with the increased granulopoiesis, despite the fact that all grafts were made to 9-day-old host embryos. On the other hand, Danchakoff noted that grafts to 9- or 10-day-old hosts stimulate erythropoiesis as well as granulopoesis. At present, no good explanation for this discrepancy can be offered.

In contrast to the attention paid by Danchakoff to the cellular nature of the response, experimental evidence relevant to the mechanism by which the response is effected is extremely meager. In one brief paragraph (1918, p. 133), the following important, yet completely undocumented, statements were made: (1) Grafted tissue must be living to produce the effect; aseptic dead tissues evoke no response. (2) Not all living tissues of the adult fowl are effective; the stimulating action seems to be a property of definite adult organs (spleen, liver, bone marrow), belonging only to the same species. These statements indicate that the effective substance is species-specific and relatively organ-specific; but no data in substantiation were presented.

Willier (unpublished), on the other hand, noted that the response of embryo spleen to grafts of fragments of adult chicken organs shows considerably less organ-specificity. In 13 out of 36 successful cases in which adult chicken thyroid fragments were grafted, the host embryos acquired greatly enlarged (7 cases) or slightly enlarged (6 cases) spleens. Furthermore, 7 adult liver grafts and 11 adult thymus grafts produced a similar response. Histological studies indicated that the observed enlargement of the spleen was due to an intense proliferation of leukocytes and to the presence of necrotic areas. The host spleen leukocytosis was related to a similar proliferation in the graft. No indications of erythropoiesis were reported.

Pomerat (1949) has confirmed the observations by the afore-mentioned workers with regard to the enormous enlargement of embryonic chick spleen following chorioallantoic grafts of adult spleen. No data are presented to indicate the specificity of the reaction, nor are any experiments reported bearing on the question of the mechanism of action. It was inferred that host spleen enlargement following homologous organ grafts is a phenomenon similar to that reported by Weiss and Wang (1941) and that a similar interpretation might be applied. This may well be the case, yet it is difficult to see how such an inference could be drawn from the facts previously reported. Danchakoff (1916) advanced no fewer than four hypotheses regarding the nature of the stimulus. Since certain of these earlier postulates played an important role in determining the scope of the present in-

vestigation, it is pertinent to consider their status in the light of the evidence presented above. Briefly, Danchakoff proposed that enlargement of the host spleen following homologous organ grafts might be explained on the basis of the following hypotheses:

1. The growth of tumor-like metastases in the host spleen, initiated by the transport, via the circulation, of graft fragments. This explanation is denied by the histological evidence presented by all investigators to date.

2. An accumulation of debris or necrotic materials, sloughed off from the graft and picked up by splenic phagocytes. This possibility is opposed not only by the available histological evidence but also by the fact that dead tissues and heteroplastic transplants are ineffective, while homologous spleen grafts, which are characteristically healthy implants, are most effective. Furthermore, the histological evidence opposes the argument that the host spleen is accumulating and lysing erythrocytes proliferated in the graft. In addition, adult fowl spleen is not actively erythropoietic either *in situ* or in grafts.

3. "A general, stimulating, chemical action" on the mesenchyme. It is impossible to ascertain the author's impression of the nature of such an action. From an analogy used to describe the type of activity observed, however, it may be inferred that Danchakoff considered a splenic hormone as a possibility. While it is maintained by some (Eddy, 1921; Downs, 1948) that the spleen does produce one or more hormones, which function in controlling the erythrocyte content of the circulation, the evidence is meager and conflicting and cannot be regarded as critical. Moreover, the fact that the stimulus is class-specific opposes the view that a nonspecific growth factor or vitamin is involved.

4. An immune reaction, in which graft antigens stimulate the production of antibodies by cells proliferated in the spleen. Although a considerable body of evidence points to the adult spleen as one site of antibody formation in the adult (Rich *et al.*, 1939; Burnet and Fenner, 1948; Fagraeus, 1948), it is almost equally well established that circulating antibodies are not formed to any extent during embryonic life. Proteins concerned with immune processes normally arise relatively late, in the last quarter of incubation or shortly after hatching (Nace and Schechtman, 1948). Wolfe and Dilks (1948) have presented evidence which indicates that over 50 per cent of newly hatched chicks tested were unable to produce precipitins against bovine serum. Other chicks similarly injected gave extremely low-titered antisera. It appears unlikely, moreover, that antibodies were produced by the embryo spleen in response to adult organ grafts of the same species but not against heteroplastic grafts. Loeb (1921), however, has argued that homoplastic grafts might incite greater host reaction than heteroplastic grafts, owing to the fact that the former are actively growing and produce toxic metabolites capable of evoking a host response, while the latter become necrotic at once and thus are incapable of producing toxins. In the present investigation this argument is opposed by the fact that, histologically, adult mouse and adult chicken spleen grafts contain similar amounts of apparently living and healthy splenic tissue. In support of the histological evidence, moreover, the following experiments have been conducted in order to determine whether antibodies against the engrafted tissues might be detected in the host blood or in the host spleen.

Several series of chorioallantoic transplants were made, in the usual manner,

to 9-day-old embryo hosts, employing the following donor tissues: (1) adult chicken spleen, (2) adult mouse spleen, (3) 9-day-old embryonic chick spleen, and (4) adult chicken brain, gonad, or heart, respectively. After the graft had developed for 8 days, each egg was opened carefully at some distance from the graft, the embryonic membranes were saturated with 2 per cent sodium citrate, a large umbilical vessel was punctured, and the blood collected in the air chamber, from which it was pipetted into 10 × 50-mm. test tubes. Approximately 2 ml. of blood were removed from each embryo. The blood was allowed to stand for 1 hour at room temperature, centrifuged for 5 minutes at 2,000 r.p.m., and the serum (or plasma, for clotting did not invariably occur because of excess citrate) was collected and stored at 4° C. The grafts were recovered and fixed for further study. Next the host spleens were removed and weighed separately on a precision balance, after being drained for 1 minute on ten sheets of filter paper. Each spleen was then sliced with a razor blade into six or eight slices, which were incubated in 1.0 ml. of 0.9 per cent NaCl at 37°.5 C. for 1 hour, and refrigerated at 4° C. for 2 additional hours, after which the mixture was homogenized in a Ten Brock tissue-grinder. The homogenate was centrifuged for 15 minutes at 2,000 r.p.m., and the supernatant fluid was then stored at 4° C., for later tests of its antibody content.

The test antigens employed were saline extracts of normal embryonic and adult chicken organs. The organs were homogenized separately in a Waring Blendor with 0.9 per cent NaCl (final concentration: 1 gm. of tissue to 5 ml. of saline), centrifuged for 15 minutes at 2,000 r.p.m., and the supernatant fluid stored at 4° C.; this supernatant was designated the "stock antigen."

The method used in determining the antibody content of host embryo blood and spleen extracts was essentially like that described by Hanks (1935) and recently applied in immunogenetic studies by Fox (1949). This technique is a highly sensitive ring-precipitin test for determining antibody in small amounts of serum. Each stock antigen was diluted with 0.9 per cent NaCl to the point at which a precipitin ring could be seen clearly and then was dissolved in 5 per cent gelatin. Approximately 0.03 ml. of each gelatin-antigen mixture was layered into the bottom of a micro-precipitin tube and allowed to harden. Over the hardened gel was layered 0.02 ml. of host embryo serum or host spleen extract. The tubes were incubated at 37°.5 C. for 1 hour and stored overnight in the refrigerator. Then, after 5–10 minutes at 37°.5 C., to remove clouding of the gelatin, readings were taken. Results were recorded as o = negative, ± = faint ring, + = definite ring. The following controls were employed: (1) positive controls, in which antibody produced in adult chickens against adult mouse spleen was reacted with the homologous antigen; (2) normal serum controls; and (3) saline controls. The sera and spleen extracts of 20 embryos bearing grafts and of 5 unoperated controls were tested in this manner with a variety of homologous and heterologous antigens. Over two hundred separate precipitin tests were checked. No evidence of antibody content in either sera or splenic extracts of embryos bearing grafts or of control embryos was obtained. All reactions were negative, with the exception of the positive controls, i.e., those between anti-mouse spleen serum from adult chickens and the homologous antigen. These results indicate that the enlargement of the host embryo's spleen following organ grafts is not correlated with antibody production per se.

One further possibility must be considered here: Can enlargement of the host spleen be a reflection of an immune response, not of a humoral, but of a strictly cellular, nature? This was suggested by the correlation reported by Willier (1924) between leukocyte proliferation in host spleen and graft and by the several investigations of Sandstrom (1932, 1936), who concluded that host-graft antagonism is dependent on the age and specificity of the grafted tissue. This postulate, however, cannot account for the class-specificity of the stimulus without recalling the hypothesis advanced by Loeb (see above), which, as the histological evidence clearly demonstrates, is not applicable here. A similar argument opposes the further possibility that increased granulopoiesis is stimulated by the "leucocytosis-promoting factor" reported by Menkin (1949) to emanate from a site of injury. It is quite unlikely that response to inflammation could exhibit the specificity encountered here.

From these considerations it is clear that none of the interpretations proffered on the basis of earlier, incomplete data is tenable when viewed in the light of the current evidence, since all the earlier postulates fail to account for the degree of class-specificity and organ-specificity demonstrated. At present, the best interpretation of the observed results is offered by the template-growth concept, viz., that substances released by the grafts catalyze the synthesis of additional molecules with configurations identical to the graft substances, resulting in enlargement of the homologous organ. This explanation best fits the data now available. It must be regarded as tentative, however, until further experiments are completed to demonstrate the relative capacities of spleen and heterologous organ grafts to evoke an enlargement of the spleens of host chicks.

ONTOGENETIC CHANGE IN THE CHICK SPLEEN AS REVEALED BY GRAFTING EXPERIMENTS

In order to determine the relative effectiveness of spleen grafts from embryonic and hatched chick donors in evoking enlargement of the host spleen, two series of transplants were analyzed, in which the donor tissue was taken from chick embryos of 9–20 days' incubation or from chicks from 1 to 49 days after hatching, respectively. The mean weight of the host spleens in each group was compared with the values previously obtained for the series of control and adult chicken spleen grafts. From the results, which are included in Table 1, it is evident that spleen grafts from progressively older donors show a parallel increase in ability to evoke enlargement of the host spleen. The mean weight of the spleens of 40 host chicks on which embryonic chick spleen had been grafted was 6.4 ± 0.32 mg., which is a substantial increase over the mean of the control series, 5.3 ± 0.13 mg., i.e., (Difference/S.E. of difference) = 3.1. When hatched chick spleen was employed as donor material, enlargement of the host spleen was even greater. The average spleen weight of 42 embryos bearing grafts of hatched chick spleen was 8.5 ± 0.52 mg. The difference between this mean and the mean value obtained in the control series is significant, i.e., (Difference/S.E. of difference) = 5.9.

Moreover, within each donor age bracket, i.e., within the embryonic and hatched chick donor groups, respectively, there is a decided trend toward an increased ability to produce enlargement of the host spleen with advancing age. This trend can be seen in Table 2. The following points are particularly noteworthy: (1) the ability to evoke a response of the homologous organ appears in the chick

spleen only in the latter third of embryonic life. Spleen grafts from embryos incubated less than 14 days are ineffective, for the mean weight of 20 host spleens following grafts of this type, 5.4 ± 0.36 mg., does not differ significantly from the mean weight of the controls. (2) Grafts from older embryonic donors (14–20 days) produce a significant enlargement of the host spleen, the mean weight of the host spleens being 7.0 ± 0.44 mg. Moreover, the trend is continued when progressively older chicks serve as spleen donors, until spleen grafts from 40–49-day-old chicks evoke enlargement of host spleens almost equal to that produced by grafts of adult chicken spleen.

TABLE 2

RESPONSE OF EMBRYONIC HOST SPLEENS TO GRAFTS OF SPLEEN FROM PROGRESSIVELY OLDER EMBRYONIC AND HATCHED CHICK DONORS

	Age of Donor			
	Embryonic		Hatched	
	8–13 Days	14–20 Days	1–20 Days	40–49 Days
No. of cases	20	20	27	15
Mean weight of host spleens (mg.)	5.4	7.0	8.2	10.5
Standard deviation	1.6	2.0	3.2	2.7
Standard error of mean	0.36	0.44	0.61	0.69

The evidence presented demonstrates a progressive increase during the normal development of the chick spleen of those factors capable of evoking enlargement of the host's spleen when grafted to the chorioallantoic membrane. Danchakoff stated (1918, p. 133) that embryonic chick tissues will grow on the allantois without producing any noticeable change in host tissues. However, since she failed to indicate the ages of chick embryos used as donors, it must be assumed that donors of less than 14 days' incubation were used, since no host spleen response was achieved. In the absence of the necessary data, no further conclusion can be drawn to explain the discrepancy. In a subsequent publication, Danchakoff and Danchakoff (1930) drew a similar conclusion from a comparison of the effects of larval or adult frog spleen grafted into the dorsal fins of tadpoles, namely, that, although adult spleen elicits an enlargement of the host spleen, larval spleen is ineffective. Unfortunately, however, the ages of the larvae used were again not given. Therefore, no comparison with the present investigation can be made.

ONTOGENETIC CHANGE IN THE ANTIGENIC SPECIFICITY OF THE SPLEEN AND OTHER ORGANS OF THE CHICK

It is apparent that the initial phase of the investigation has raised far more questions than have been solved. The striking progressive increase in a class-specific, relatively organ-specific component of the chick spleen, capable of evoking a characteristic enlargement of the homologous host organ when grafted to the chorioallantoic membrane, forms a solid groundwork for further experimental analysis. It is now possible to consider the nature of the factors involved. Among the pertinent questions to be considered are the following: What biochemical changes occur in the chick spleen

during the latter half of embryonic development which may be correlated with the observed increase in its capacity to elicit a response of the host spleen? More specifically, considering the concept of growth held by Weiss and Tyler, we may ask what changes occur in the protein constituents of the spleen during this period. Are the factors concerned localized in any particular splenic fraction, e.g., can differences in activity be detected in nuclear and cytoplasmic elements? As the first step in the analysis of the chemical nature of the factors concerned, the development of saline-soluble organ antigens of the spleen has been studied by means of precise immunological techniques. In addition, a parallel investigation of heart and brain antigens has been conducted.

EXPERIMENTAL PROCEDURE

Preparation of antigens.—Healthy adult chickens, of several common breeds and of both sexes, were used as the source of adult organ antigens for injection and test purposes. Saline extracts of spleen, brain, heart, and liver were prepared according to the following standard procedure, aseptic technique being employed. Fowls were first bled by cardiac puncture (the 30–60 ml. of sterile blood collected was citrated and refrigerated for later use in absorption tests), and then decapitated, and the afore-mentioned organs were removed rapidly to large, individual Stender dishes of cold 0.9 per cent NaCl. The organs were sliced and were washed in three changes of saline over a period of 2 hours in the refrigerator. Tissue slices were then drained on sterile filter paper, weighed, and mixed with sterile saline. For each gram of tissue, 5 ml. of saline solution were added. Tissues were then homogenized in a Waring Blendor; during this procedure the temperature was not allowed to rise above 10° C. The resultant homogenate was refrigerated for 3–6 hours and then centrifuged for 30 minutes at 2,000 r.p.m. The slightly cloudy supernatant extract was used undiluted for injections into rabbits and served as stock antigen for further dilutions when used in absorptions and precipitin tests. All injections, absorptions, and serological tests were made with freshly extracted antigens, except for certain preliminary experiments in which the activity of frozen and frozen-dried antigens was tested. Despite considerable evidence to the contrary (Maltz *et al.*, 1948; Schechtman, 1948), frozen and frozen-dried organ extracts proved to be less sensitive than freshly prepared antigens; therefore, the latter were used throughout the course of work.

White Leghorn embryos of 9, 12, and 18 days' incubation (at 37°.5 C.) were used as sources of the embryonic organ antigens used in absorptions and precipitin tests. The method of extraction was essentially the same as that described for adult antigens, with one exception: organs were ground in a Ten Brock Pyrex tissue-grinder and were then made up to the final concentration (1 gm. of tissue in 5 ml. of saline solution). This departure from standard procedure was necessitated by the small total volume used in these extractions. It is of interest to note the number of embryos necessary to supply a usable amount of antigen: 20 at 18 days; 30 at 12 days; and 40 at 9 days.

Antigens from organs of adult mice were used in determining the class-specificity of antisera produced against antigens from chicken organs and were extracted from the organs of adult C57 strain mice, of both sexes, following the procedure described for antigens from organs of adult chickens.

Antibody production and collection. All injections were made into healthy, adult, male albino rabbits. In each case, prior to

the first injection, a preliminary bleeding was made from the marginal vein of the ear. Blood was collected in 60-ml. centrifuge tubes, allowed to clot at room temperature for 1–2 hours, stored in the refrigerator overnight, and centrifuged at 2,000 r.p.m. for 5–10 minutes. The serum collected was sterilized by passage through a Seitz filter and was stored at 4° C. in serum bottles until needed for control tests.

Three groups of rabbits were injected with sterile saline extracts of adult chicken spleen, heart, or brain, respectively. Four rabbits were injected with spleen extracts, and three each with extracts of heart or brain. Each rabbit received a primary course of five injections over a 10-day period. The first four injections were given intravenously and consisted of 0.5, 1.0, 2.0, and 3.0 ml. of organ extract, respectively. The fifth injection consisted of 1.0 ml. given intravenously and 3.0 ml. intraperitoneally. A trial bleeding at 7–10 days after the fifth injection usually indicated a low-titered antiserum; thus, as standard procedure, each rabbit was rested at least 3 weeks after completion of the primary injection series and was then given an anamnestic injection consisting of 2.0 ml. intravenously and 3.0 ml. intraperitoneally. Seven days later, rabbits were bled and the sera collected, sterilized, and stored as described above. Although a few rabbits showed signs of anaphylactic shock, none was lost owing to the use of the anamnestic reaction.

Absorption of antisera.—Prior to use in serological tests, all sera, both normal (control) and experimental, were subjected to the following treatment: (1) inactivation of complement by heating to 56° C. for 30 minutes and (2) complete absorption with whole adult chicken blood (pooled blood from at least three fowls). The absorptions were made as follows: 0.5 ml. of full-strength serum was mixed with 0.3 ml. of whole blood, incubated at 37°.5 C. for 1 hour, and refrigerated for at least 6 hours. The serum was cleared by centrifugation and was retested with whole blood. Absorption was continued in this manner until the serum no longer reacted.

From a consideration of the composite antigenic nature of the organ extracts injected and from the observations of previous investigators (Kidd and Friedewald, 1942; Landsteiner, 1945; Maculla, 1948*a*), it appeared likely that both control and antiorgan sera would cross-react with heterologous organ antigens at low dilutions. This proved to be the case. Therefore, it was necessary to absorb antiorgan sera with each test antigen separately to achieve the specificity essential in determinations of ontogenetic change in antigenicity. Absorptions were carried out as described for whole-blood absorption, except for the fact that, initially, 0.5 ml. of serum was combined with 0.5 ml. of antigen, which in each case consisted of the stock antigen, diluted 1:10,000. This antigen dilution was used because preliminary titrations indicated that it fell within the equivalence zone or, in some cases, in the zone of slight antibody excess. This eliminated the danger of inhibition by excess antigen. Absorption was continued until no further reaction was obtained upon retesting.

Precipitin tests.—Determinations of organ- and class-specificity of sera were made by the serum-dilution titer method, in which a series of progressive serum dilutions was set up and tested with a fixed quantity of antigen. This is the classical method of assaying the potency of an antiserum and is critical, provided that the antigen is not present in large excess. To avoid inhibition due to excess antigen, a preliminary titration was made with a constant volume of full-strength serum

and progressively decreasing amounts of antigen. The end-point, or least amount of antigen giving a good precipitate, was then used with serial dilutions of serum. This technique, which was described by Martin (1943), is critical, since the serum end-point titer is greatest with the least effective amount of antigen (cf. Kabat and Mayer, 1948). Five-tenths ml. of antigen (stock antigen diluted 1:5,000–1:10,000, as indicated by preliminary titration) was added to each of eleven tubes (75 × 10 mm.) containing 0.5 ml. of serum diluted over a range from full strength to 1:1,024. All dilutions were made with 0.9 per cent NaCl. Readings were made after 30 minutes at 37°.5 C. and after 12 hours at 4° C. The following controls were set up: antigen-saline, saline-antiserum, antigen-normal serum.

Precipitin tests of absorbed antisera in combination with embryonic test antigens were made as follows: 0.05 ml. of test antigen, in the indicated effective dilution, was mixed with an equal volume of serum, diluted 1:8 with saline, in 50 × 5-mm. tubes. Readings were made as indicated above, and similar controls were tested.

SPECIFICITY OF ANTISERA

The production of class- or species-specific and organ-specific antisera is a subject of theoretical and practical importance. In the past, numerous experiments have been undertaken with this end in view. Antiorgan sera have been evaluated: (1) by injections into animals of the donor and other species, in attempts to produce cytotoxic effects *in vivo;* (2) by analysis of their effects of cultures of homologous and heterologous tissues *in vitro;* and (3) by serological tests (Landsteiner, 1945). While varying opinions have been expressed with regard to the specificity of action, considerable evidence now favors the view that antiorgan sera are generally class-specific but that most sera of this type display only partial organ-specificity (Harris, 1948*a*).

Table 3 shows the effective titers of normal and antichicken organ sera when reacted with homologous and heterologous antigens of the adult chicken and mouse. (In these experiments, "titer" is taken to be the highest active serum dilution capable of reacting in precipitin tests with standard saline-soluble antigen, prepared as previously indicated.) In this table each value represents the average titer obtained from tests of sera from three rabbits of each group, each test being run in duplicate. It should be noted that all normal sera tested react, at low dilutions, with extracts of both chick and mouse tissues. Normal (natural) antibodies are found in the sera of most animals (Landsteiner, 1945); however, the highest titer obtained in these experiments falls slightly below that usually reported, 1:32 (Duran-Reynals, 1939; Kidd and Friedewald, 1942). This may be accounted for by the fact that all sera were absorbed with whole blood before testing. Further inspection of the table reveals that antichicken organ sera do not react with homologous or heterologous mouse organ antigens at significantly higher titers than those of normal serum, the highest titer recorded being 1:8. These results clearly show that the antiorgan sera tested are highly class-specific. Organ-specificity, however, is only partial. Each antiserum reacted most strongly with homologous organ antigen; a lesser reaction was obtained with heterologous organ antigen, however, which was greater than may be attributed to natural antibodies present.

The results obtained in the present investigation are in agreement with the findings of others, in so far as the class- and organ-specificity of antiheart and antispleen sera are concerned (Pomerat,

1947; Harris, 1948; Maculla, 1948*a*); but the results obtained with antibrain sera contradict certain earlier reports (Bailey and Gardner, 1940; Henle, Chambers, and Groupé, 1941; Lewis, 1941; further references in Cooper, 1946). Schechtman (1948) found that rabbit antisera against extracts of brain of 18-day-old embryo chick reacted not only with extracts of brain but also with liver, heart, and muscle extracts as well, Cross-reactivity was not appreciably diminished by absorption which chicken blood. There were no distinct quantitative differences in reactivity which might indicate relative organ-specificity. As noted in the discussion of the preparation of antigens, however, fresh antigens were used for both injections and test purposes in the present investigation. Schechtman, on the other hand, used lyophilized antigens throughout the course of the work reported. This technical difference may well account for the observed variance in results, for preliminary tests in this laboratory indicate lyophilized cellular antigens to be less sensitive in the precipitin reaction.

Grunwaldt (1949) has recently reported that antisera prepared in rabbits against extracts of brain from hatched chicks inhibit cellular outgrowth in tissue cultures of 9- and 13-day-old embryonic chick spinal cord but are only slightly inhibitory to cultures of 21-day-old rat embryo brain and have no effect on guinea pig (75 mm. C.R.) or human embryo (22 mm. C.R.) brain cultures. This evidence appears to support the conclusion that antichick brain sera are relatively class-specific. Grunwaldt, however, apparently influenced by earlier reports of the lack of class-specificity in antibrain sera, concluded: "These observations do not contradict the investigations previously reported, since it has been proven that the rat and guinea pig do not acquire antigenicity until after birth. . . ." This conclusion seems unwarranted, for it is based on the commonly accepted misconception that embryos are devoid of antigens. There is little doubt that both quantitative and qualitative differences in antigenic composition exist in embryonic and adult organs (Burke *et al.*, 1944; Maculla, 1948*c*), but a considerable number of an-

TABLE 3

SPECIFICITY OF ANTICHICKEN ORGAN SERA BY PRECIPITIN TESTS
(Reciprocal of Highest Effective Serum Dilution)

Test Antigen		Serum*				
Extracts of		Saline	Nomal	Antispleen	Antiheart	Antibrain
Saline		.....	.....	.....	.....	.....
Adult chicken	Spleen	.....	8	512	16	128
	Heart	.....	8	64	1,024	64
	Brain	.....	16	64	32	512
	Liver		4	4	8	32
Adult mouse	Spleen	.....	2	2	4	8
	Heart	.....	4	2	4	2
	Brain	.....	4	4	4	2
	Liver	.....	2	2	8	2

* Complement inactivated, absorbed with whole chicken blood.

tigenic substances has been shown to be identical in embryo and adult. Moreover, there is no critical evidence that embryonic proteins are less antigenic than those of the adult (cf. Cooper, 1948, pp. 427–28).

ONTOGENY OF SALINE-SOLUBLE ANTIGENS OF SPLEEN, BRAIN, AND HEART

Embryonic development is characterized by increasing specialization and increasing levels of complexity. In the production of the antigenic complex of the adult organ or tissue, has development proceeded from a pool of "common" antigenic substances toward the more highly specialized antigens of each tissue? Do the "common" antigens represent cellular constituents which are more primitive and unspecialized than are organ-specific antigens? The following experiments have been directed toward answering these questions. While the investigation has centered on the development of antigens of the spleen, experiments have been conducted concomitantly on heart and brain. These parallel studies provide two valuable comparisons: (1) Since the spleen is both histologically and functionally a compound organ, which makes its appearance relatively late in development and undergoes several marked changes in its histogenesis, it was desirable to compare its antigenic development with that of an organ which appears considerably earlier and which exhibits a more direct line of cell specialization, e.g., the heart. (2) Of the cross-reactions obtained with antispleen sera, the strongest were those with brain extracts; thus a comparison is possible of the development of specific and common antigens of the two organs. In addition to extracts of adult organs, antigens from three embryonic stages were chosen for analysis, viz., 9, 12, and 18 days of incubation. These choices were made with regard to the development of the spleen at the periods indicated. Thus extracts of spleen from 9-day-old chicks were chosen, since at that time the spleen undergoes a period of granulopoiesis and potential erythropoiesis; moreover, all hosts for chorioallantoic grafts were incubated 9 days, and, in addition, a large group of graft donors were taken at 9 days. Spleen extracts from 12-day-old chicks were analyzed for two reasons: (1) this stage falls immediately before the period of lymphocyte formation, and (2) it also immediately precedes the rise in spleen growth on day 13, described by Latimer (1928). Extracts of spleen from 18-day-old chicks were studied as representative of the period of lymphocyte formation.

On the basis of the preceding experiments, the two antisera in each group which showed the highest titer with homologous antigen were selected for further tests. Each antiserum was absorbed, first with whole chicken blood and then with either homologous or heterologous antigen of a specified stage. Aliquots of the absorbed serum were then tested separately with each homologous and heterologous antigen from all stages (9, 12, 18 days and adult). The procedure may be seen most clearly by an inspection of Table 4, a summary of results obtained when two antispleen sera were tested as described. Each test was run in duplicate. Parallel series of tests were made with antibrain and antiheart sera, and the data were compiled in a similar manner.

The generalized results obtained from over fifteen hundred separate absorption tests are presented schematically in Figure 1 and may be summarized as follows: (1) Saline extracts of 9-, 12-, and 18-day-old embryonic heart contain at least two active antigens or combining groups which are identical, or closely related, to antigens of the adult heart. Of these, one

antigen is organ-specific; the second antigen is held in common with 18-day-old and adult brain. (2) Saline extracts of 9-, 12-, and 18-day-old embryo chick brain contain one antigen or combining group identical with, or closely related to, an adult antigen. This fraction is brain-specific. Two additional, separate, antigenic components are found in 18-day-old and adult brain, which are not present in extracts of 9- or 12-day-old brain. One of these antigenic groups is common to 9-, 12-, 18-day-old, and adult heart; the other to 9-, 12-, 18-day-old, and adult spleen. (3) Saline extracts of 9-, 12-, and 18-day-old chick spleen contain, in addition to the fraction held in common with 18-day-old and adult brain, a spleen-specific fraction, S1, identical with, or closely related to, an antigen in adult spleen. In this respect, spleen extracts resemble similar preparations of heart. However, a second spleen-specific antigen or combining group, S2, is found in extracts of 18-day-old and adult spleen. This is clearly shown by reciprocal absorption tests (see Table 4). Antispleen sera absorbed with adult or 18-day-old spleen extracts no longer react with spleen antigens from any stage tested. On the other hand, absorption with 9- or 12-day-old embryonic spleen antigens removes only the capacity to react with 9- or 12-day-old spleen extracts; such absorbed sera still react with 18-day-old and adult extracts.

These results, which show clearly that a second spleen-specific antigen appears during the latter third of embryonic development, are especially provocative when it is recalled that it is also during

TABLE 4

ANTISERA AGAINST SALINE EXTRACTS OF ADULT CHICKEN SPLEEN TESTED AGAINST SALINE EXTRACTS OF 9-, 12-, AND 18-DAY-OLD EMBRYONIC AND ADULT CHICKEN ORGANS

Serum	Test Antigens																
Antispleen serum absorbed with whole chicken blood, followed by absorption with:	Spleen				Heart				Brain				Liver				Saline
	9	12	18	Ad.	9	12	18	Ad.	9	12	18	Ad.	9	12	18	Ad.	
None	+	+	+	+	o	o	±	±	o	o	±	±	o	o	o	o	o
Spleen extract 9	o	o	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Spleen extract 12	o	o	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Spleen extract 18	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Spleen extract ad.	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Heart extract 9	+	+	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Heart extract 12	+	+	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Heart extract 18	+	+	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Heart extract ad.	+	+	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o
Brain extract 9	+	+	+	+	o	o	±	±	o	o	o	o	o	o	o	o	o
Brain extract 12	+	+	+	+	o	o	+	+	o	o	o	o	o	o	o	o	o
Brain extract 18	o	o	±(o)	±(o)	o	o	o	o	o	o	o	o	o	o	o	o	o
Brain extract ad.	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Liver extract 9	+	+	+	+	o	o	±	±	o	o	±	±	o	o	o	o	o
Liver extract 12	+	+	+	+	o	o	±	±	o	o	±	±	o	o	o	o	o
Liver extract 18	+	+	+	+	o	o	±	±	o	o	±	±	o	o	o	o	o
Liver extract ad.	+	+	+	+	o	o	±	±	o	o	±	±	o	o	o	o	o

this period that the spleen first acquires the ability to elicit enlargement of the host spleen when grafted to the chorioallantoic membrane. No conclusions may be drawn, however, until additional experiments are completed. This work, now in progress, should reveal, more precisely, the time between the twelfth and ninth day of development. Two common antigens, however, did not appear in brain extracts until after day 12, for they were demonstrated only in extracts from 18-day-old and adult chickens. These antigens were held in common with heart and spleen, respectively, and appeared in the latter organs as early as day 9. These

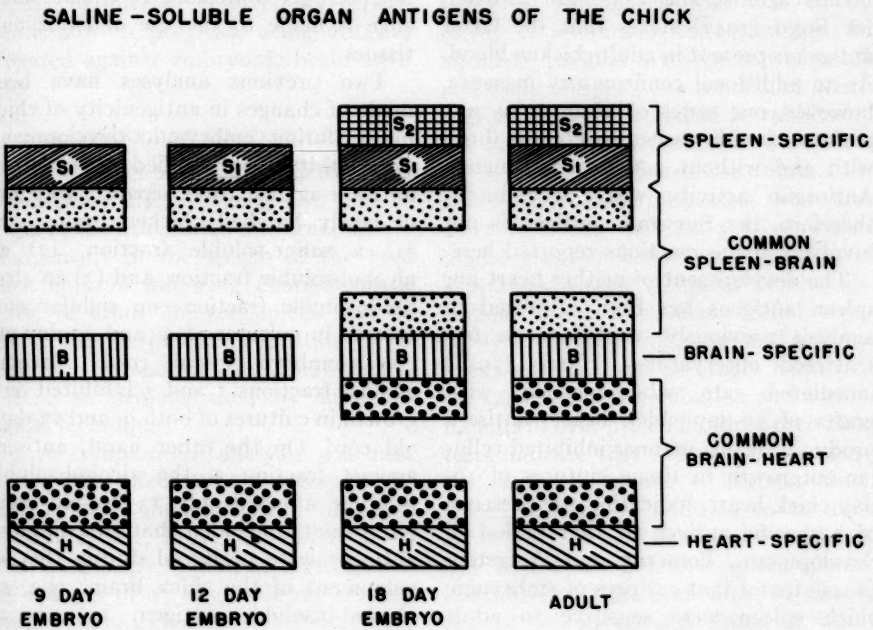

FIG. 1

eighteenth days of incubation at which antigen S2 can first be detected. Moreover, experiments are planned which should establish whether or not a causal correlation exists between the appearance of antigen S2 and the development of the capacity to effect an increase in the size of the homologous organ.

Also of interest is the finding that the development of "common" antigens does not necessarily precede the formation of specific antigens. Brain-specific antigen, identical with, or closely related to, adult brain antigen, was found as early as the facts suggest that the widespread distribution of an antigen or combining group does not necessarily indicate that it appears earlier in ontogeny than does an antigen localized to a particular organ.

It is quite clear that the saline-soluble organ antigens involved in the present study are neither the blood cell or serum antigens nor the Forssman antigen, for absorption of antiorgan sera with whole chicken blood does not remove the specific antibodies in question. Since several recent studies have established the occurrence of individual genetic differences

in antigenic constitution of fowl erythrocytes (Briles, McGibbon, and Irwin, 1948), whole chicken blood, pooled from at least three fowls, was used in all absorptions. This precaution minimized the possibility of cross-reactivity due to individual differences. Whole-blood absorption also served to remove antibodies against the Forssman antigen, for Boyd (1947) states that the latter antigen is present in adult chicken blood. As an additional confirmatory measure, however, one series of absorptions was performed with washed sheep cells (both with and without added complement). Antiorgan activity was not reduced; therefore, the Forssman antigen is not involved in the reactions reported here.

The development of neither heart nor spleen antigens has been subjected to analysis previously, except for a few scattered observations. Harris (1948) inoculated rats subcutaneously with grafts of 16-day chick heart; antisera produced in this manner inhibited cellular outgrowth in tissue cultures of 16-day chick heart, indicating the presence of a specific antigen at that period of development. Pomerat and Anigstein (1944) found that cultures of embryonic chick spleen were sensitive to adult reticulo-endothelial immune serum (REIS = antispleen + bone-marrow serum), which points to the presence of antigens common to embryonic and adult spleen. However, since the organ-specificity of REIS is not strong and absorptions were not carried out, strict comparisons cannot be made. Maculla (1948*c*) compared the antigenic compositions of adult and embryonic mouse organs; three antigenic fractions were analyzed—saline-soluble, saline-insoluble, and nucleoprotein; all tests were done by means of the complement-fixation technique. She observed that antisera against adult mouse spleen did not react with embryonic mouse spleen and concluded that adult and embryonic spleens have different antigenic constitutions. Evaluation of this work must await the publication of further details, which are almost completely lacking in the preliminary report. To cite one instance: the ages of embryos used have not been given, although frequent reference is made to the use of embryonic tissues.

Two previous analyses have been made of changes in antigenicity of chick brain during embryonic development. Grunwaldt (1949) studied the effects of antisera against three separate fractions of newly hatched chicken brain—viz., (1) a saline-soluble fraction, (2) an alcohol-soluble fraction, and (3) an alcohol-insoluble fraction—on cellular outgrowth in cultures of 9- and 13-day-old chick embryo spinal cord. Antisera against fractions 1 and 3 inhibited outgrowth in cultures of both 9- and 13-day-old cord. On the other hand, antisera against fraction 2, the alcohol-soluble fraction, affected only 13-day-old cord. Grunwaldt concluded that two different antigens were produced during the development of the chick brain; one, an alcohol-insoluble antigen, appears as early as day 9; the second, which is alcohol-soluble, develops between the ninth and thirteenth days of incubation. Burke *et al.* (1944) presented evidence for two saline-soluble brain antigens, one of which occurs in embryos from 160 hours to at least 312 hours, and a second, which appears at all stages after 260 hours. While these two investigations are in agreement regarding the time of origin of two brain-specific antigens, one is puzzled by the reported difference in the nature of the second antigen. It is possible, of course, that two antigens—one alcohol-soluble, one saline-soluble—appear between days 9

and 13. The results of the present investigation agree with those of both Burke *et al.* and Grunwaldt, in that a brain-specific antigen is present as early as day 9. It is, however, identical with, or closely related to, an adult brain antigen and is not transitory, like the antigen described by Burke *et al.* These differences may be resolved by experiments now in progress, using antisera prepared against embryonic brain antigens.

SUMMARY AND CONCLUSIONS

1. The object of this investigation was to determine the specificity and time of origin of the substances concerned in the enlargement of the spleen of the host chick embryo following chorioallantoic grafts of adult chicken spleen and to relate the responsible substances, if possible, to specific antigens of the spleen, as demonstrated by immunological techniques.

2. A comparative analysis was first made of the effects on the spleen of the host embryo due to grafts of adult chicken spleen, heart, or gonad or of adult mouse spleen. The mean weight of the spleens of 17-day embryos bearing grafts of adult chicken spleen was over 100 per cent greater than the mean weight of the spleens of embryos receiving only a drop of saline. Grafts of adult mouse spleen, on the other hand, produced no enlargement of the host's spleen. In contrast to the class-specificity of the effect, only a partial organ-specificity was exhibited.

3. Grafts from embryonic chick donors of less than 14 days' incubation were not effective in producing enlargement of the host's spleen. Spleen grafts from older embryonic donors (14–20 days' incubation) produced a significant increase in weight of the host's spleen, however. Furthermore, the capacity to evoke enlargement of the host's spleen increased with donor age, up to 7 weeks, posthatching.

4. As a further step in the analysis of the nature of the factors concerned, the development of saline-soluble organ antigens of the spleen was studied, by means of immunological techniques. In addition, parallel investigation of heart and brain antigens was conducted. Anti-organ sera were produced in rabbits by the injection of saline extracts of adult chicken spleen, heart, or brain, respectively. Each antiserum was first absorbed with an extract of either homologous or heterologous organs of a specified stage. Aliquots of the absorbed serum were then tested separately with each homologous and heterologous antigen from all stages employed (9, 12, 18 days of incubation and adult). Results of over fifteen hundred precipitin tests indicate that saline extracts of 9-, 12-, and 18-day-old embryonic chick spleen contain, in addition to an antigenic fraction held in common with 18-day and adult brain, a spleen-specific fraction (S_1), identical with, or closely related to, an antigen found in adult spleen. In this respect, spleen extracts resemble similar preparations of heart. However, as shown by reciprocal absorption methods, an additional spleen-specific antigen or combining group (S_2) is found in extracts of 18-day and adult spleen.

5. These data suggest a correlation between the progressive increase with age in the ability of spleen grafts to evoke enlargement of the host's spleen and the differentiation, during a similar period in ontogeny, of a specific group of spleen antigens.

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THE CONCENTRATION OF RIBOFLAVIN IN THE TISSUES OF THE EMBRYOS, LARVAE, JUVENILES, AND ADULTS OF THE FROG, RANA PIPIENS PIPIENS

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MANY investigations have shown that riboflavin is present in most animal tissues. Other studies have clearly demonstrated its function as a unit in prosthetic groups of specific coenzymes in some intracellular respiratory systems. In spite of the extensive study of riboflavin in cancer research and other investigations, little work in addition to the Texas studies (Taylor *et al.*, 1942; Snell and Strong, 1941) seems to have been done on the comparative amounts of this vitamin in various vertebrate tissues. The only available publications pertaining to developmental changes have been three determinations for the rat (Murray *et al.*, 1946). The present paper shows the variations in the concentration of riboflavin in the tissues of the embryos, larvae, juveniles, and adults of the frog, *Rana pipiens pipiens* Schreber; it contrasts the concentration of this vitamin in tissues of adult frogs in March with those in August and September, and the results for tissues of the adult frogs in August and September are compared to the concentration of riboflavin in corresponding tissues of the rat and man.

The reasons for selecting *R. pipiens*

[1] This research was completed in partial fulfilment of the requirements for the degree of Doctor of Philosophy under the direction of Dr. Joseph Hall Bodine, head of the Zoölogy Department, State University of Iowa, Iowa City, Iowa. The writer will be teaching at Eastern Illinois State College, Charleston, Illinois, after September 1, 1950.

pipiens for this study are as follows: (1) it has been used extensively as a laboratory animal; (2) it was available in its natural habitat; (3) during its development it adjusts to the change from mostly herbivorous to carnivorous feeding habits; and (4) its physiological compensation for stresses of hibernation during the winter may be studied.

MATERIAL AND METHODS

The embryos, larvae, juveniles, and adults of *R. pipiens pipiens* were taken from several neighboring ponds south of Iowa City, Iowa, not more than 24 hours prior to their analysis. The stages of the embryos were classified according to Shumway (1940) and the larvae as listed by Taylor and Kollros (1946).

The assaying procedure as outlined by the Association of Vitamin Chemists, Inc. (1947), was used with slight modifications. Specimens were killed by bleeding, as the blood contained fluorescing fractions of iron, porphyrin, and other pigments. If present, parasites were removed from each sample with the aid of a binocular dissecting microscope; each sample was weighed to the nearest milligram, then finely ground with a plunger test-tube homogenizer. With the exception of the eyes, which required re-extraction, a quantity of 0.25 N sulphuric acid was added (Bodine and Fitzgerald, 1947) such that less than 5 per cent of the total amount of riboflavin obtained in the first extraction was procured in the second assay. After the homogenized tissue and solvent had been placed in an amber bottle, the solution was autoclaved for 15 minutes under 15 pounds' pressure. The solution was incubated at 45° C. for 12 hours. Following incubation, the solution was centrifuged at high speed and the debris removed; small droplets of fat on the solutions containing liver and ovary were removed by aspiration. A 2.5 N sodium acetate solution was used to decrease the acidity of the solution to 4.5 pH; at this acidity there was very little fluorescent intensity change for ±1 pH unit (Scott *et al.*, 1946).

Sixteen ml. of the solution at pH 4.5 which contained the extract of the tissue were placed in a cuvette, and a reading was taken on the galvanometer of a Pfaltz and Bauer fluorophotometer, to obtain the total fluorescence (for a description of this instrument see Bodine and Fitzgerald, 1947). Standardization of the fluorophotometer was obtained by the methods of Hodson and Norris (1939). Approximately 10 mg. of sodium hydrosulphite crystals were added to the solution in the cuvette to obtain the reducible fraction due to riboflavin's fluorescence. Concentrated sodium hydroxide was added to another cuvette containing a solution of the tissue's extract, so that the fluorescence might be observed at pH 10.5, as riboflavin does not fluoresce at this pH, interfering fluorescing pterines and related compounds were measured if they were present (Bodine and Fitzgerald, 1947). If the reading at pH 10.5 was higher than the nonreducible portion (the fluorescence which could not be reduced by sodium hydrosulphite) at pH 4.5, it was assumed that pterines which reduced the same as riboflavin at pH 4.5 had been formed (Burgess, 1949). Of the tissues used, only the eye presented this problem. The reading at pH 10.5 for the eye was subtracted from the total galvanometer reading to obtain the fluorescent fraction due to riboflavin. Procedures and calculations as described by Bodine and Fitzgerald (1947) were performed for each determination. One ml. of a riboflavin solution containing 0.4 γ/ml was added to a cuvette containing 15 ml. of a solution of the tissue's extract, and each determination was corrected for

the percentage recovery of the added riboflavin.

Preliminary tests for the deterioration of riboflavin due to light were conducted. A clear 6-ounce bottle and a similar 6-ounce amber bottle were both filled with a standard solution of riboflavin (0.1 γ/ml); an equal volume was poured from each bottle daily, and the fluorescence was measured. The bottles were placed on the laboratory table and kept at 70° F. from March 1 to March 9. The laboratory contained two south windows, which were darkened by brown blinds over the lower three-fourths and by white paint over the upper portion. The riboflavin in the clear bottle deteriorated very rapidly; little fluorescence remained after 9 days of exposure. Fluorescence in the amber bottle decreased less than 8 per cent in 8 days (see Fig. 1).

Between embryonic stage 25 and lar-

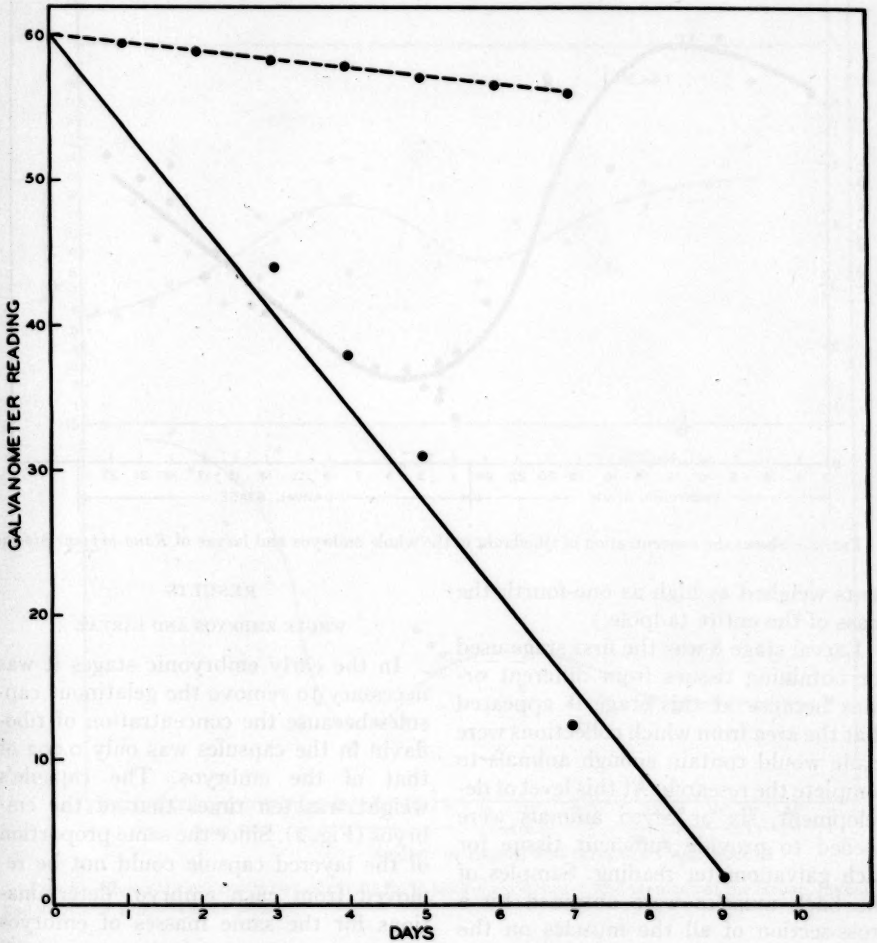


FIG. 1.—Shows the results of a deterioration test of a standard solution of riboflavin (0.1γ/ml.) in a 6-ounce amber bottle (*broken line*) and a similar clear-glass bottle (*solid line*).

val stage 1 the tadpoles began feeding. After feeding had commenced at larval stage 1, the intestines were removed because mud and debris in the solution increased turbidity and prevented an accurate reading for assays in Figure 2. (Although the gut and its contents were found to contain only about one-tenth the concentration of riboflavin that was in the animal proper, the gut and its contents weighed as high as one-fourth the mass of the entire tadpole.)

Larval stage 8 was the first stage used for obtaining tissues from different organs because at this stage it appeared that the area from which collections were made would contain enough animals to complete the research. At this level of development, six or seven animals were needed to provide sufficient tissue for each galvanometer reading. Samples of the back muscles were obtained by a cross-section of all the muscles on the vertebral column, for during the early larval stages individual muscles did not provide sufficient tissue for determinations.

All results have been recorded in γ/gm of wet weight of tissue. The Association of Vitamin Chemists, Inc. (1947), states that results by this general method can be duplicated to within 5–10 per cent. Curves were drawn by the writer as nearly as possible to the means of the nearest determinations (Figs. 2, 3, 4, 5, and 6).

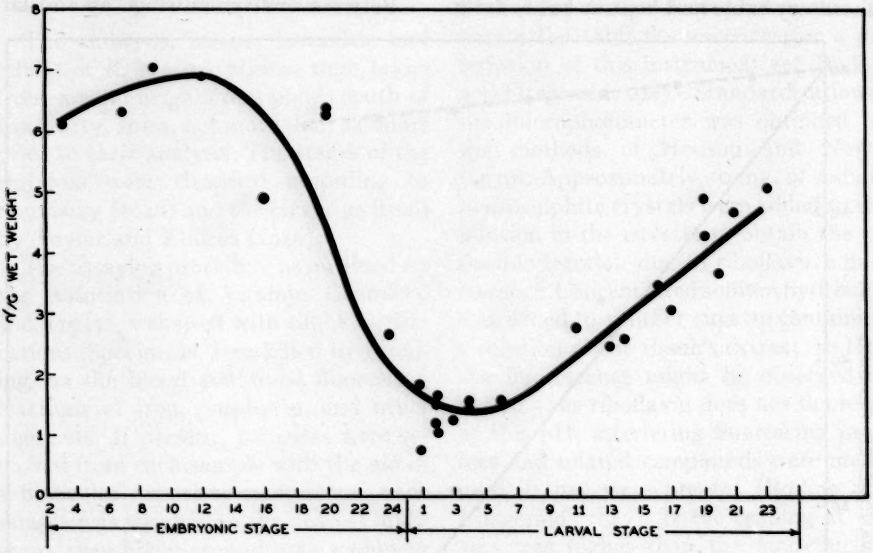
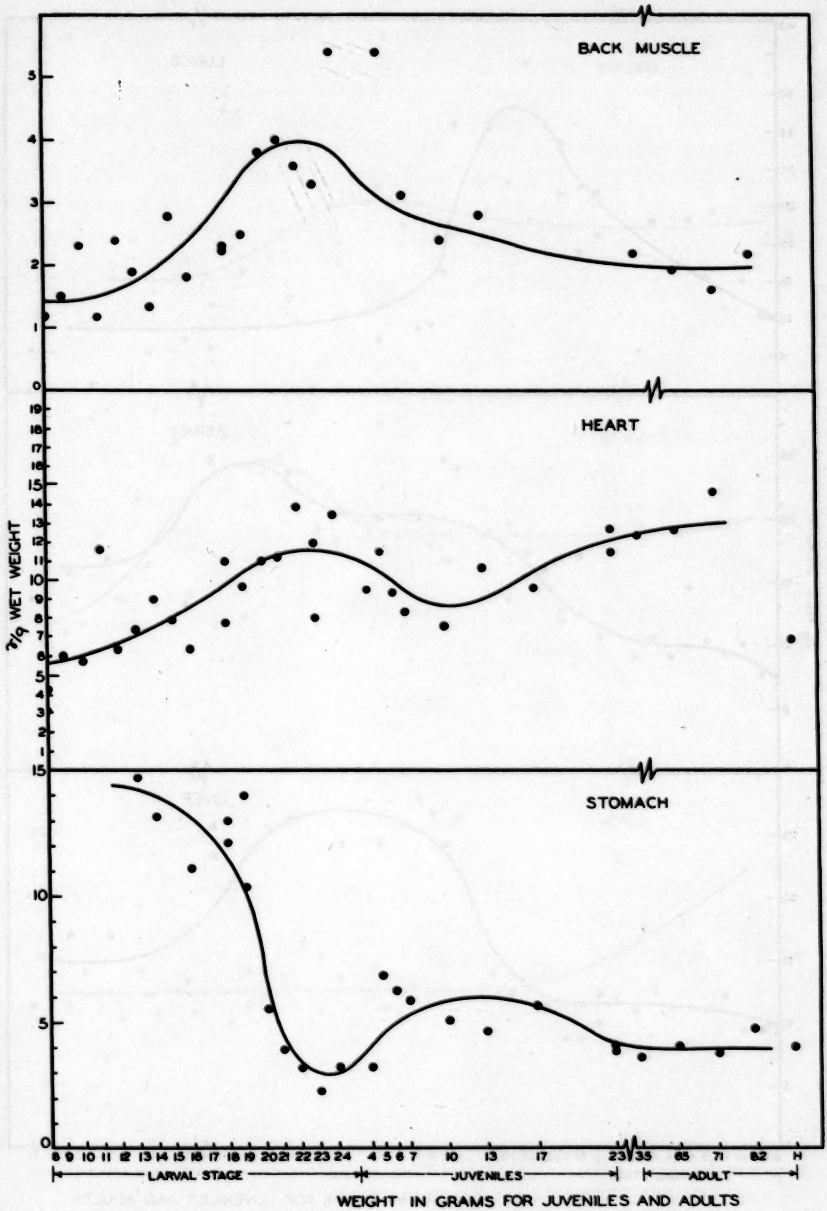


FIG. 2.—Shows the concentration of riboflavin in the whole embryos and larvae of *Rana pipiens pipiens*

RESULTS

WHOLE EMBRYOS AND LARVAE

In the early embryonic stages it was necessary to remove the gelatinous capsules because the concentration of riboflavin in the capsules was only 0.002 of that of the embryos. The capsule's weight was ten times that of the embryos (Fig. 2). Since the same proportion of the layered capsule could not be removed from each embryo, determinations for the same masses of embryos varied as much as 1 γ; therefore, in all embryonic stages up to stage 20, only the


BACK MUSCLE
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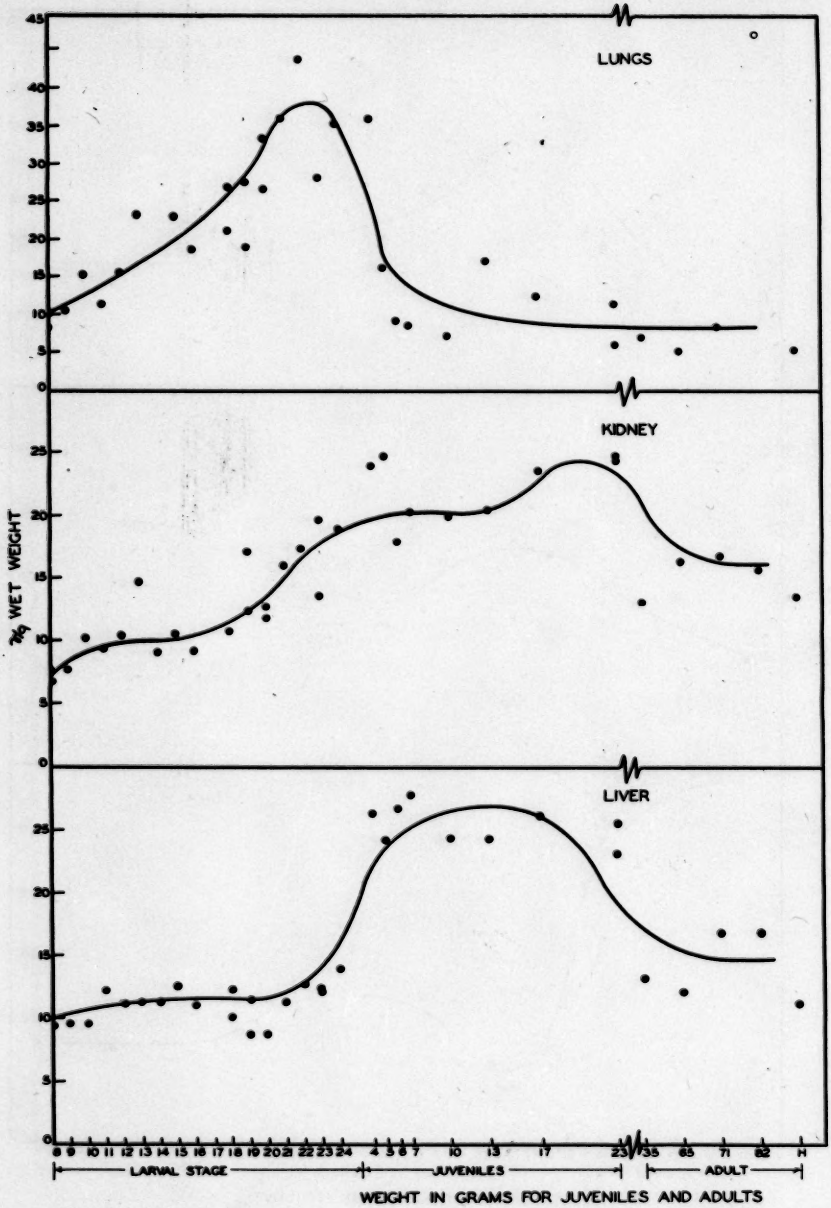
LARVAL STAGE
JUVENILES
ADULT
WEIGHT IN GRAMS FOR JUVENILES AND ADULTS

FIG. 3.—Shows the concentration of riboflavin in back muscle, heart, and stomach of the larvae, juveniles, and adults of *Rana pipiens pipiens*.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LARVAL STAGE
JUVENILES
ADULT
WEIGHT IN GRAMS FOR JUVENILES AND ADULTS

FIG. 4.—Shows the concentration of riboflavin in the lung, kidney, and liver of the larvae, juveniles, and adults of *Rana pipiens pipiens*.

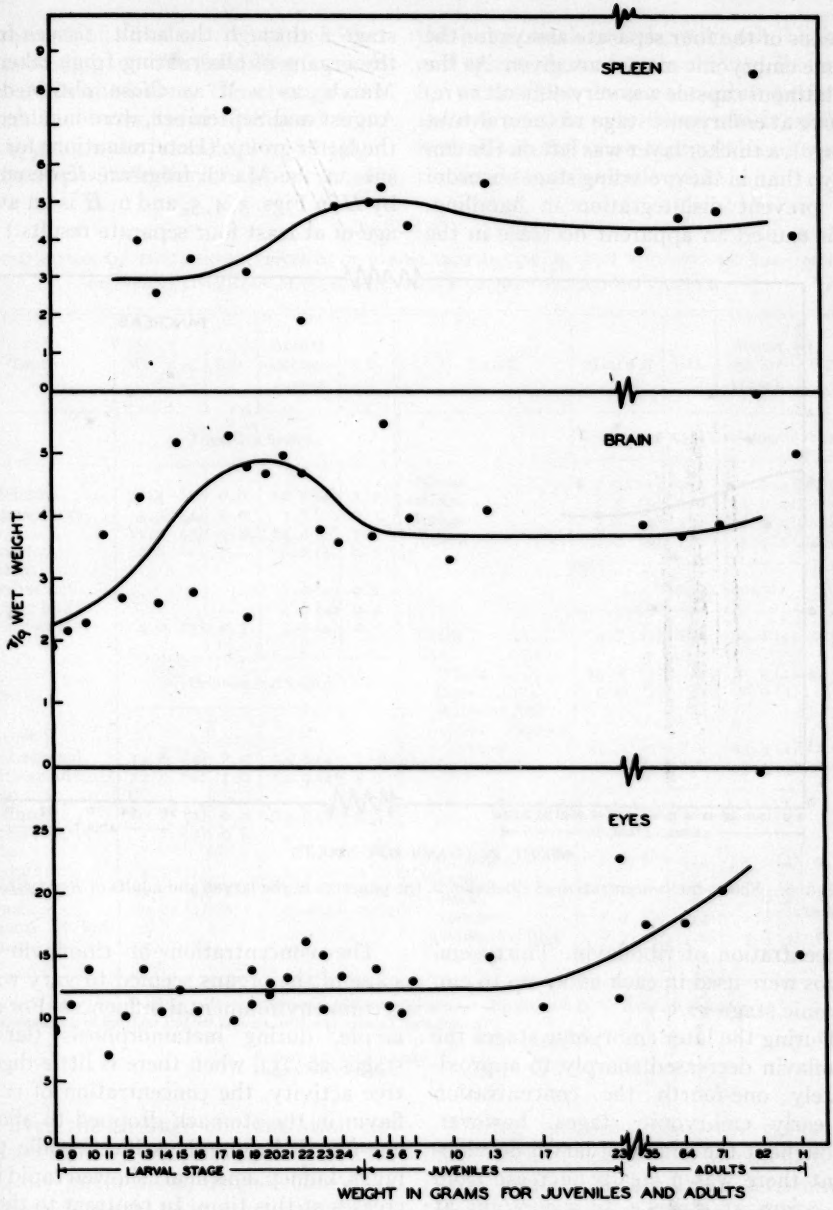

FIG. 5.—Shows the concentration of riboflavin in the spleen, brain, and eyes of the larvae, juveniles, and adults of *Rana pipiens pipiens*.

means of the four separate assays for the same embryonic masses are given. As the gelatinous capsule was very difficult to remove at embryonic stage 16 (neural-tube stage), a thicker layer was left on the embryo than in the preceding stages in order to prevent disintegration in handling; this caused an apparent decrease in the stage 8 through the adult; tissues from the organs of hibernating frogs taken in March, as well as those obtained in August and September, were included in the latter group. (Determinations for tissues of the March frogs are represented by *H* in Figs. 3, 4, 5, and 6; *H* is an average of at least four separate results.)

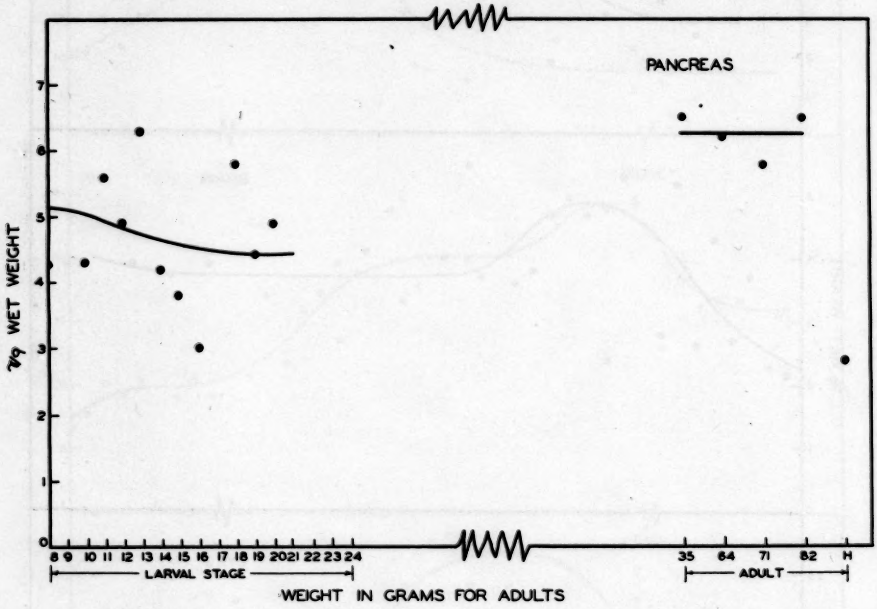

FIG. 6.—Shows the concentration of riboflavin in the pancreas of the larvae and adults of *Rana pipiens pipiens*.

concentration of riboflavin. Thirty embryos were used in each assay up to embryonic stage 22.

During the later embryonic stages the riboflavin decreased sharply to approximately one-fourth the concentration of early embryonic stages, however, throughout the course of larval development there was a steady increase from 1.5 γ/gm at stage 1 to 5.0 γ/gm at stage 23.

DETERMINATIONS FOR TISSUES OF LARVAL, JUVENILE, AND ADULT STAGES

The tissues from corresponding organs were assayed for riboflavin from larval

The concentration of riboflavin in some of the organs seemed to vary with certain environmental influences. For example, during metamorphosis (larval stages 20–25), when there is little digestive activity, the concentration of riboflavin in the stomach dropped to about one-fourth its previous level, while the lungs, kidney, and heart showed rapid increases at this time. In contrast to these results, the eyes and liver remained at a constant level during metamorphosis as well as during the previous larval period.

During the juvenile stage, only the liver and kidney exhibited higher con-

centrations of riboflavin than in either the preceding or the following periods (Fig. 4). In the kidney the average concentration of riboflavin decreased from 22.0 γ/gm for the juveniles to 15.6 γ/gm for the adults, a decline of 29 per cent. In the lungs, back muscle, stomach, brain, and spleen showed little change. Except for an increase during the adult period, the eye was the only tissue which exhibited about the same mean value during the larval and juvenile periods.

TABLE 1*

A COMPARISON OF THE CONCENTRATION OF RIBOFLAVIN (γ/GM OF WET WEIGHT) IN *Rana pipiens pipiens* TISSUES IN MARCH WITH THOSE OF AUGUST AND SEPTEMBER

Tissue	March *H*	S.D.	August and September	S.D.
Digestive System				
Duodenum	7.4 (4)	0.8	10.1 (4)	1.7
Intestine, large	3.5 (4)	1.0	2.7 (2)	0.1
Liver	11.2 (5)	3.4	14.8 (4)	2.1
Pancreas	2.8 (1)	.....	6.2 (4)	0.3
Stomach:				
Cardiac half	.....	.....	3.0 (4)	0.2
Pyloric half	.....	.....	4.8 (4)	0.2
Whole	4.0 (5)	0.7	3.9 (4)	0.2
Urinogenital System				
Kidney:†				
And adrenal	13.8 (4)	2.5	14.8 (4)	1.5
Without adrenal	12.5 (2)	1.0	14.9 (4)	1.9
Ovary:				
Mature	10.8 (2)	0.8	12.8 (4)	0.4
Immature	5.7 (2)	0.8	.....	.....
Testis:				
Mature	5.7 (1)	.....	3.4 (2)	0.2
Immature	2.5 (4)	0.4	2.6 (4)	0.5
Oviduct	0.61 (4)	0.1	1.6 (4)	0.05
Adenoma in kidney	3.9 (1)	.....	.....	.....
Respiratory and Circulatory Systems				
Blood	0.81 (5)	0.1	1.1 (4)	0.7
Heart	7.5 (5)	1.0	13.1 (4)	1.0
Lungs	5.9 (5)	2.9	7.2 (4)	1.4
Spleen	4.7 (4)	1.0	5.4 (4)	1.8
Nervous System				
Brain	4.7 (5)	1.2	4.4 (4)	0.9
Eye:				
Whole	15.8 (1)	.....	22.5 (4)	6.9
Lens	0.0 (4)	.....	0.0 (4)	.....
Without lens and vitreous humor	29.1 (5)	8.2	43.4 (4)	12.0
Spinal cord	3.9 (4)	1.1	3.7 (4)	0.1
Miscellaneous Tissues				
Bone, femur	1.8 (5)	0.4	3.1 (4)	0.4
Muscle:				
Back	.....	.....	2.0 (4)	0.2
Gastroc.	1.9 (6)	0.4	1.8 (5)	0.4
Tendon, Achilles'	2.5 (1)	.....	2.3 (4)	0.4
Tongue	2.2 (5)	0.3	1.8 (4)	0.1

* The number in parentheses following the means indicates the number of assays. "S.D." is standard deviation.

† Comparison of the right and left kidneys, with and without the adrenals:

	35 Gm.	31 Gm.
Kidney without adrenal	12.4	15.4
Kidney with adrenal	12.0	14.5

the liver the average concentration decreased from 25.3 γ/gm for the juveniles to 14.8 γ/gm in the adults, a decline of 42 per cent. It would be interesting to know whether or not the fall in the concentration of riboflavin in the liver and kidney is a factor contributing to senescence in the frog.

During the juvenile and adult periods during metamorphosis the pancreas diminished in size to such an extent that it was impossible to make a determination from the available pancreatic tissue.

ADULT TISSUES TAKEN IN MARCH CONTRASTED WITH THOSE OF AUGUST AND SEPTEMBER

All the results in Table 1 fall roughly into three groups: the very rich tissues of

special functions, such as the eye, liver, kidney, and mature ovary, varied in concentration from 10 to 61 γ/gm; an intermediate, highly diversified group, including the brain, spinal cord, spleen, lungs, stomach, and pancreas, ranged in value from 3 to 7 γ/gm; the low-valued aggregation contained striated muscle and the various connective tissues with results below 3 γ/gm. In all three groups most of the results for tissues taken during hibernation in March were lower than those of August and September.

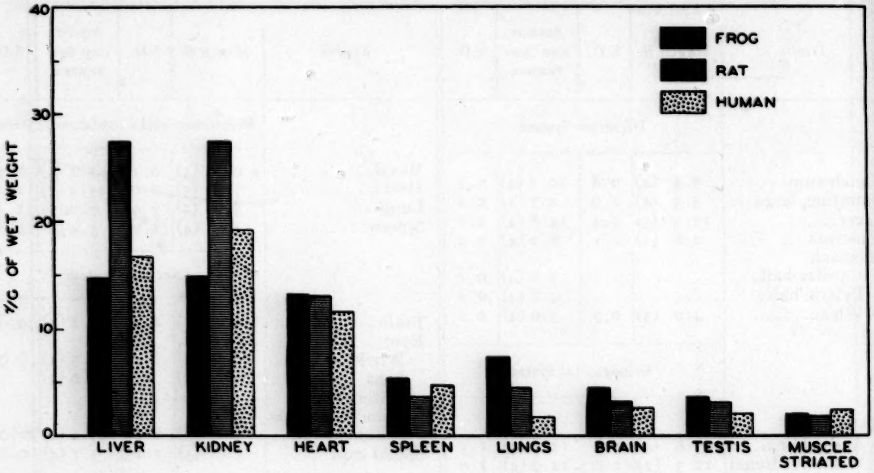

FIG. 7.—Shows a comparison of the concentration of riboflavin in frog, rat, and human tissues. The results for the rat and human tissues were determined by the microbiological method (Taylor *et al.*, 1942). The rat tissues were taken from a 190-gm. male and a 220-gm. female from a Wistar rat colony. The human tissues are an average of two females and one male between the ages of twenty-five and forty-two who died from stab and gunshot wounds.

A COMPARISON OF FROG, RAT, AND HUMAN TISSUES

Thirteen of the results of Taylor *et al.* (1942) are within 43 per cent of the determinations made on frog tissues (Fig. 7). Some of these variations may be attributed to the use of a different method of assay (microbiological) for the rat and human tissues. The concentration of riboflavin in the cardiac muscle exhibited the least percentage variation of all the tissues shown in Figure 7; the results for the rat and frog are about equal, while the assays for human heart muscle are within 10 per cent of the concentration in the frog. In a comparison of results from tissues of the frog and man the determinations for the lungs showed the widest percentage variation. In the rat, only the liver and kidney exhibited more than 39 per cent variation from the concentration of riboflavin in the frog; possible explanations of this deviation in the liver are given in the discussion.

DISCUSSION

If the concentrations of riboflavin at various stages of development are compared, such results should be indicative of the portion of intracellular respiration mediated through the flavoproteins. Lardy *et al.* (1949) states: "Given two alternative metabolic pathways, with enzymes for both pathways present, the course of metabolism will depend in part

upon the relative amounts of the respective enzymes." However, the total physiological effect of metamorphosis, hibernation, and other environmental influences is not completely understood; thus the writer may only conjecture as to the cytological and histological changes which have caused the variations in concentration of riboflavin (Figs. 2–6).

Between embryonic stage 12 and larval stage 1 the concentration of riboflavin dropped to almost one-fourth its previous level in the whole embryos (Fig. 2). It is during this time that muscular tissue is being formed (first muscular response at stage 18); as striated muscle contains about one-third the concentration of riboflavin found in early embryonic stages, a decrease is anticipated. As the first chromatophores appear at embryonic stage 21 (Shumway, 1942), the riboflavin may have been changed into another form or pigment as found in the grasshopper (Bodine and Fitzgerald, 1947, 1948). The gradual rise in concentration of riboflavin in the whole animal from larval stage 6 to stage 23 is probably a replenishment from the diet following the depletion during the late embryonic period.

The riboflavin in the back muscle of the frog had an average concentration of 2.0 γ/gm, and the gastrocnemius 1.8 γ/gm (Table 1). Mickelsen *et al.* (1939) found that round steak from cattle contained 1.9 γ/gm, and Taylor *et al.* (1942) determined that the striated muscle of the rat contained 1.8 γ/gm, and the average of three human muscle tissues was 2.2 γ/gm. If the concentration of riboflavin in the striated muscle of the backs of juvenile frogs is compared with that of 30-day-old rats and the adult frogs compared with yearling rats, little difference is noted (Murray *et al.*, 1946). A comparison of the above muscles with some of the other tissues in Figure 7 shows a narrow range in the concentration of riboflavin in the same types of tissues taken from animals widely separated phylogenetically.

Only the tissues of the heart and eye exhibited higher concentrations of riboflavin in the adult than in the juvenile frogs. The decrease in the concentration of the adult heart from 13.1 γ/gm during August and September to 7.5 γ/gm in March was one of the most significant drops of all tissues contrasted at these two periods; this decrease coincides with the reduced heart output and metabolic rate of hibernation.

In the early larval stages of the frog the stomach consists chiefly of a very thin tube of mucosa and serosa. The decrease during larval stages 18–21, when muscle layers were being added to the stomach, indicated that riboflavin was probably located in the highest concentration in the mucosa. (Assays of smooth muscle of the digestive tract ranged from 2 to 3 γ/gm.) The supposition that the mucosa is high in riboflavin is also verified when the averages of the cardiac and pyloric halves of four different stomachs are compared (Table 1); the cardiac half contains a larger volume of mucosal secretory area than does the pyloric half. The inactivity of the stomach during metamorphosis may account for the low concentration of riboflavin when compared with previous and following stages (Fig. 3).

In the lung tissue the rapid increase from 10 γ/gm at larval stage 8 to 35 γ/gm during metamorphosis indicated that riboflavin played an important rôle in lung respiration in tadpoles. The sharp decline following metamorphosis perhaps signifies that the part played by this vitamin in the lungs has changed from a dominant to a minor role in the early juvenile frogs. As can be seen in Figure 4, wide variations were encountered in lung

tissue. The open circle of 47.2 γ/gm given for the 82-gm female frog was the result of an average of 45.3 γ/gm for the right lung and 49.1 γ/gm for the left. Since this was more than six times the average of the other adult lung tissues, it was not included in Table 1. As all visible parasites, such as *Rhabdias bufonis*, were removed under the dissecting lens, the high value can hardly be attributed to a large portion of parasites; however, a pathological condition is possible, though the lungs grossly appeared normal. Wide variations were encountered by Snell and Strong (1941) in their assays of rat lung.

Sjostrand (1946) observed, by means of fluorescent microphotographs of the kidney of the frog *R. temporaria*, that this vitamin seemed to lie chiefly in the basal rod structures of the proximal convoluted tubules. Although this is an area of many mitochondria, he does not state that the fluorescence is due to their presence.

If the concentration of riboflavin is the limiting factor, the rise in riboflavin in the kidney during metamorphosis (larval stages 20–25) should increase the ability of the flavoprotein system to handle the protein degradation products from the resorbed tail. During this period the animal changes from herbivorous to carnivorous feeding habits. Since riboflavin is concerned with energy release, a comparison of the larval stages preceding stage 19 with the juvenile period would suggest that the excretion of end-products from plant tissues requires less energy via the flavorprotein system than do those of a high protein intake. The kidney tissue of larval stages 10–18 contained about half the concentration of riboflavin found in the juvenile stages.

The right and left kidneys, both with and without the adrenal gland, were assayed to determine whether the adrenal glands were extremely high (36–72 γ/gm) in concentration of riboflavin, as found by Nollet and Raffy (1940) in the frog *R. viridis*, or lower than kidney tissue (1.4–13 γ/gm), as observed by Fountaine and Callamand (1947) in mammals (see Table 1). The results of this study agree with the latter.

In a comparison of the concentration of riboflavin in the livers of 30-day-old and 360-day-old rats, Murray *et al.* (1946) found a 30 per cent increase for the older males and a 10 per cent increase for older females. This does not agree with the decrease shown in the livers of the adult frogs in Figure 4; however, the following investigations may indicate some causes of differences in concentration. Sarett and Perlzweig (1943) believed that the level of this vitamin in the body is maintained at the expense of the liver. Hodson (1940) found that high riboflavin diets resulted in a large increase in the concentration of riboflavin in the livers of chickens. Thiamine-deficient rats showed large increases in the liver's concentration of riboflavin (Luecke *et al.*, 1944).

The spleen was chosen as an example of tissue from the reticulo-endothelial system of the frog. Hibernation does not seem to produce a significant change in the concentration of riboflavin in the spleen (Table 1). Determinatons for tissues from the rat spleens were within 70 per cent of the results for the frog; the human assays of the spleen were within 85 per cent of the frog. These results would indicate that riboflavin in the spleen performs a limited function which is not greatly affected by environmental influences.

The concentration of riboflavin in the juvenile and adult stages of the brain exhibited little variation; no explanation can be given for the erratic results of the early larval stages (Fig. 5). In a comparison of the results for the brains of the

three smallest adults with those of the hibernating frogs, the latter, of all the tissues studied, shows the highest percentage increase over the determinations made in August and September.

The eyes exhibited the most consistent concentrations of riboflavin of the ten tissues which were examined during development. The slight increase in the adult may be attributed to a change in the proportion of the coats of the eye to the lens and vitreous humor; the two latter tissues have exceedingly small amounts of this vitamin.

Sjostrand (1946) found no fluorescence in the pigmented epithelium, rods and cones, or ganglion cells; however, he found intense yellow-green fluorescence in the choroid coat of *R. temporaria*. This may be contrasted with the results of Von Euler (1934), who found the retinas of fish eyes rich in flavins.

The crystalline lens contained some material which was highly fluorescent; yet there was no reducible fluorescence at pH 4.5. Fisher (1940) reported riboflavin in the lens, though Von Euler (1939) was unable to detect any riboflavin in this organ.

SUMMARY

1. The fluorometric method has been used in a study of the riboflavin content (γ/gm of wet weight) of thirty tissues in the frog, *R. pipiens pipiens* Schreber, during hibernation and early autumn.

2. The concentration of riboflavin varied widely in different tissues of the frog; yet it was remarkably constant when corresponding tissues were compared in the frog, rat, and man.

3. The content of various tissues ranges from no riboflavin in the crystalline lens to 43.4 γ/gm in the coats of the eye.

4. Most tissues exhibited decreases during hibernation, ranging from a very small amount up to a 43 per cent decline in cardiac muscle.

5. The average concentration of riboflavin in the *R. pipiens pipiens* egg up to embryonic stage 12 is 6 or 7 γ/gm of wet weight.

6. In a comparison of various stages of development, the concentration of riboflavin changed with certain external environmental influences.

7. Of the ten tissues studied during development, no two were found to follow exactly the same pattern of variation.

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INITIATION OF CELL DIVISION IN THE ARBACIA EGG BY INJURY SUBSTANCES[1, 2]

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CELLULAR injury can cause the initiation of cell division in neighboring cells. Thus, for example, Massart (1898), Haberlandt (1914), and many others have found that plant cells are stimulated to divide by injury to cells in the vicinity. Bloch (1941) has given an excellent review of the subject of wound healing in plants. Regenerative growth in invertebrates and in vertebrate organs, such as the liver, is dependent on injury; healing of wounds has been attributed partly to injury. In tissue cultures, according to Fischer (1930), a little injury to tissue in which growth has stopped brings about immediate growth of new cells, which not only causes healing of the wound but stimulates growth of the whole culture as well. In the field of cancer research the importance of injury in initiating neoplasms has been emphasized many times. Menkin (1941), for example, states that the diversity of methods and agents which induce neoplasms suggests that the final result is not a direct influence of the agent but an effect on the cell, which, in turn, as a result of injury or metabolic disturbance, may liberate a growth-promoting factor (see also Wolbach, 1925; Deelman, 1927; Howes, 1946; and Bourret and Fraisse, 1948, for discussion of the role of injury in cancer).

Apparently, injury substances are involved, but little is known as to their

[1] This paper constitutes a thesis presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

I am deeply indebted to Dr. L. V. Heilbrunn for suggesting this problem and for his kind help and guidance during the progress of this work.

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nature and mode of action. This study was begun in an attempt to find out more about these substances.

It is suggested that injury substances act like other agents which incite cells to divide, in that they cause an increase in protoplasmic viscosity. It has been found that when a cell is undergoing mitosis, very definite changes in viscosity take place. Heilbrunn and Wilson (1948), in the most complete work of the kind, made viscosity determinations with accompanying cytological studies, minute by minute, in the *Chaetopterus* egg. They found that the viscosity rises during the prophase and reaches a peak just before the appearance of the spindle. As the spindle is formed, the viscosity drops abruptly and then, just before division takes place, rises again rapidly. Similar changes have been described for other cells (see Heilbrunn and Wilson, 1948, for a discussion of this literature). It is believed that these changes in viscosity are essential for the division of the cell. According to Heilbrunn (1943), all agents, including fertilization, which can initiate cell division in the sea-urchin egg, produce an increase in viscosity. With these facts in mind, the effect of injury substances on the viscosity of the sea-urchin egg was investigated in this study.

MATERIAL AND METHODS

The sea-urchin egg has long been standard material for the study of the initiation of cell division, and it seemed of interest to inquire whether these cells could be made to divide by exposure to substances produced by injured cells. Accordingly, the eggs of *Arbacia punctulata* were used.

Injury substances were prepared from frog muscle (*Rana pipiens*) and clams (*Venus mercenaria*) according to the method described by Heilbrunn *et al.* (1946) in their studies of heat death. Tissues were cut up into distilled water and extracted at 40°–50° C. for several hours. Toluene was placed over the surface to prevent bacterial growth. The extract was then filtered to remove tissue fragments, rapidly brought to a boil to precipitate proteins, and filtered. The filtrate was then evaporated to dryness, the residue taken up in absolute methyl alcohol, and this solution filtered. The alcohol extract was then evaporated to dryness, and the residue taken up in distilled water. The evaporations were done over a hot plate with a fan blowing over the surface of the solution. In this way the process was accelerated and the temperature kept at 40°–50° C. The final solution was filtered and the tonicity adjusted with distilled water to slightly less than that of sea water. This is of importance, since sea-urchin eggs are activated by hypertonic solutions. The extracted material from 100 gm. of tissue in 30 cc. of distilled water was the approximate final dilution. In every case, the pH of the final solution was between 4 and 5. This is in line with other observations of the formation of acid following injury.

The extract was then tested for its parthenogenetic activity in the following dilutions: 5 parts of extract to 5 parts of sea water; 4 parts of extract to 6 parts of sea water; and 3 parts of extract to 7 parts of sea water. Extreme care was taken to prevent contamination with sperm. The sea urchins were washed in running tap water prior to opening them and inducing shedding, and all experiments were carried out with sea water which had been filtered and allowed to stand overnight in a glass-stoppered bottle. Untreated control eggs kept in this sea water showed no cleavages. A medicine-dropperful of concentrated egg suspension (0.8 cc.) was added to 10 cc. of the diluted extract. At various intervals 2 drops of eggs (0.1 cc.) were re-

moved, washed in 10 cc. of sea water, and placed in 5 cc. of sea water. At about 4 hours after exposure the percentage of dividing eggs was determined.

Viscosity measurements were made by the centrifuge method with an Emerson hand centrifuge. The handle was turned once per second. At this centrifugal force (about nine thousand times gravity) normal eggs stratified in 20–30 seconds. A determination of the time required to stratify the normal egg was made for each batch used, both at the beginning and at the end of the experiment. There was no change in viscosity of the normal untreated egg during the experiment. Eggs were considered stratified when the granules had been centrifuged into one half of the cell and the other half of the egg was clear. The greater the viscosity, the greater is the time of centrifugation that is necessary to stratify the eggs.

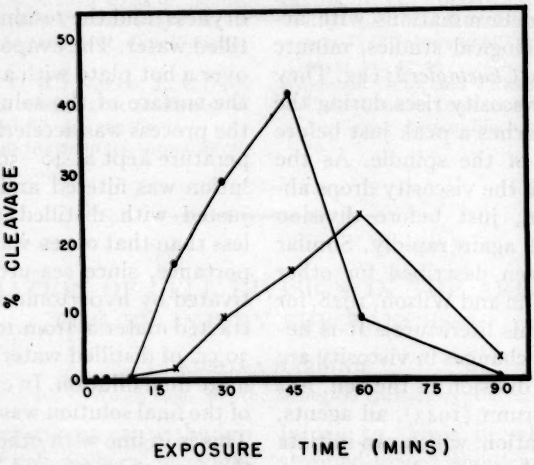

FIG. 1.—Per cent of cleavage with different lengths of exposure to the extract. Circles represent eggs exposed to a mixture of 5 parts of extract to 5 parts of sea water. Crosses represent eggs exposed to a mixture of 4 parts of extract to 6 parts of sea water.

RESULTS

Every extract that was tried caused parthenogenesis, at one length of exposure or another. Exposure of the eggs to the extract for very short times (2 or 5 minutes) had little or no effect. With longer exposures the percentage of cleaved eggs first increased, then suddenly dropped off, eventually reaching zero with the longest exposures. Figure 1 shows the results of a typical experiment. The per cent of cleavage is plotted against the time of exposure to the extract. As can be seen, this percentage increases with length of exposure up to an optimum time, then falls off with longer exposures. The counting of the number of dividing eggs was, in some cases, somewhat difficult. There were always eggs which had cytolyzed and showed small protrusions on the surface. These were not counted as dividing cells. Only those cells which had clearly divided were counted as divisions (see, e.g., Fig. 2). Few of the eggs showed regular cleavage, but some showed fairly regular cleavage up to the four-cell stage (Fig. 3). In many of the divided cells nuclear material could be detected in each of the

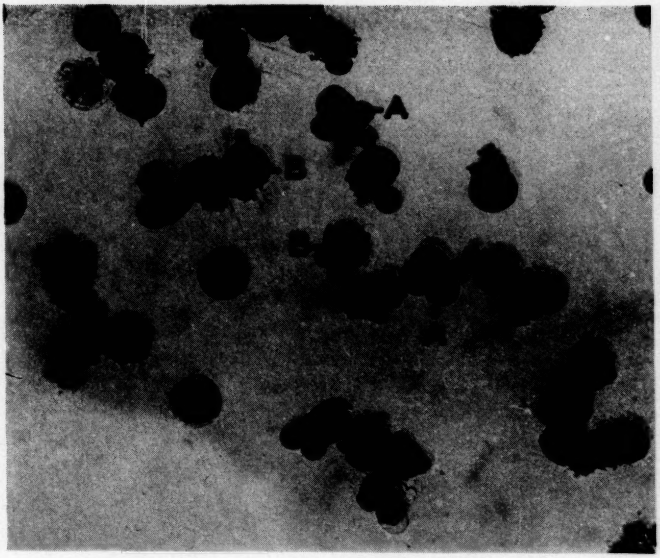

FIG. 2.—Cell divisions resulting from treatment of *Arbacia* eggs with extracts of injured tissues. *A* indicates eggs which have divided; *B* indicates eggs which are cytolyzed.

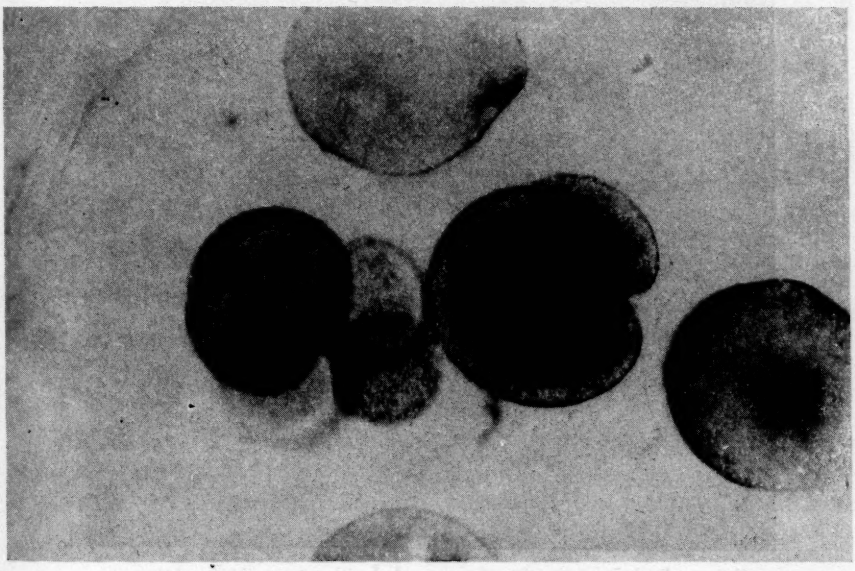

FIG. 3.—Cell division resulting from exposure of *Arbacia* eggs to extracts of injured tissues

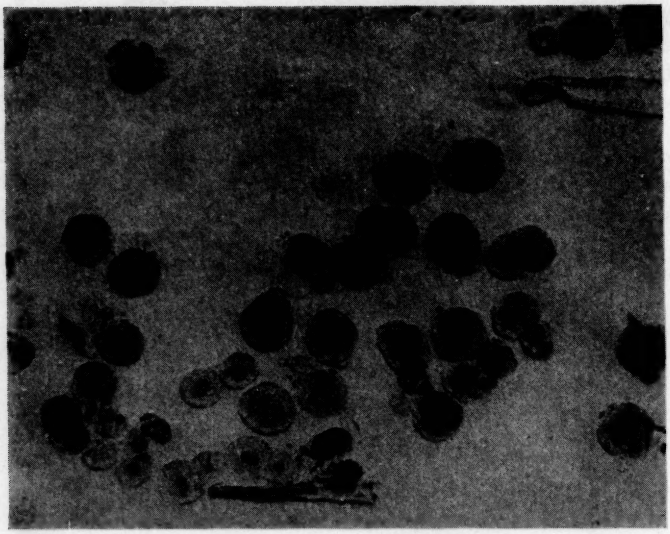

FIG. 4.—Feulgen preparation of eggs treated with extract of injured tissues

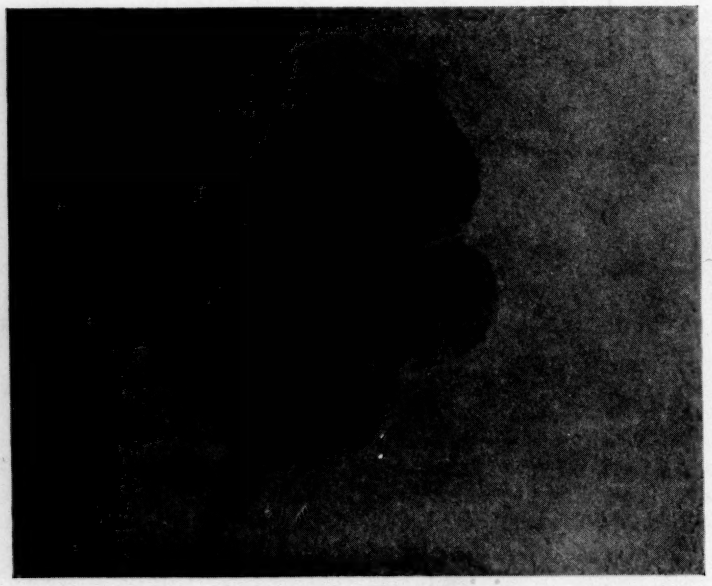

FIG. 5.—Feulgen preparation of eggs treated with extract of injured tissues

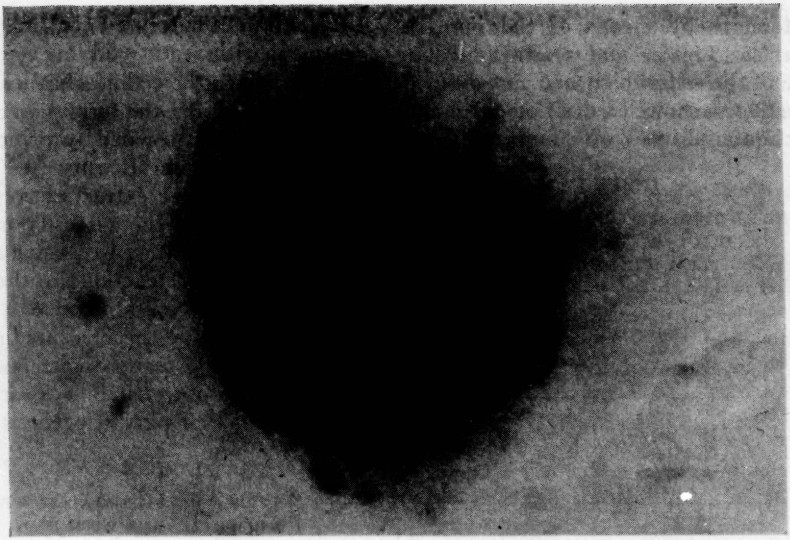

FIG. 6.—Feulgen preparation of eggs treated with extract of injured tissues

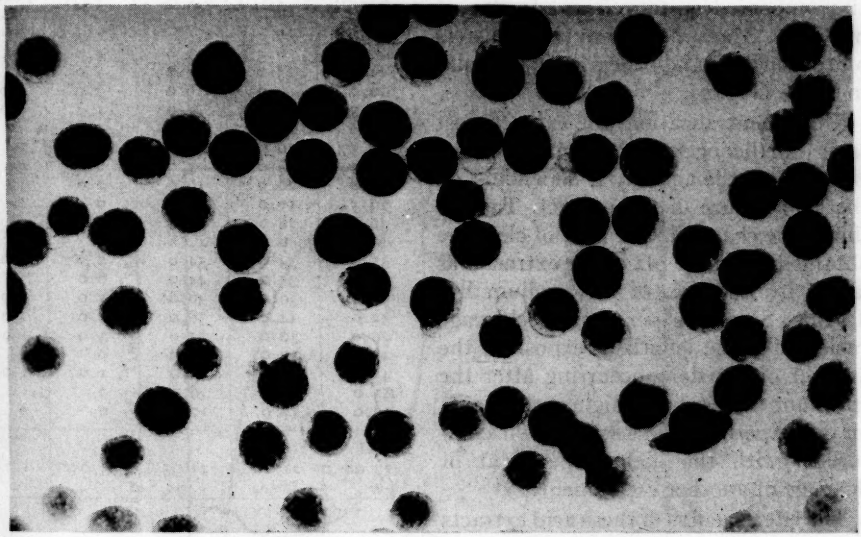

FIG. 7.—Appearance of eggs which had been exposed to the extract for too long a time

blastomeres by means of the Feulgen stain (see Figs. 4 and 5). In addition, some of the cells which had not divided nevertheless showed a division of the nuclear material into two clumps (Fig. 6).

TABLE 1

THE EFFECT OF RAISING THE pH OF THE EXTRACT ON THE PER CENT OF CLEAVAGES OBTAINED

pH of Original Extract	Highest Per Cent of Cleavage	pH of Extract after Addition of NaOH	Highest Per Cent of Cleavage
4.91.....	61.3	6.95	0
4.38.....	53.5	5.88	8.5
4.48.....	34.0	6.88	0
4.50.....	34.0	6.93	0

The whole-mount method of A. R. Whiting (1950) was used. The eggs which had been exposed to the extract for too long a time appeared quite different from normal eggs. Many had large clear blisters on the surface, and others were irregular in shape (Fig. 7).

Upon neutralization, the extracts lost their parthenogenetic activity. Apparently, then, the activity is dependent on the acid nature of the extract. Table 1 shows the change in per cent of cleavage obtained when the pH of the extract was raised by addition of 1 N sodium hydroxide. Since the per cent of cleavage varies with the length of exposure, the number of divisions occurring after the optimum time (or the highest per cent) for one experiment was selected for comparison with the highest per cent of cleavage of another experiment.

The effectiveness of these acid extracts was compared with the effectiveness of various acids at the same pH. The results are summarized in Table 2. The eggs were more severely injured by treatment with these acids than with the extracts. Figure 8, for example, shows a considerable number of cytolyzed eggs following treatment with acetic acid.

According to the colloid-chemical theory of cell division, the formation of the mitotic spindle is preceded by an increase in the protoplasmic viscosity (see Heilbrunn, 1943). These extracts have been shown to increase the viscosity of the *Arbacia* egg, in addition to causing a surface precipitation reaction in the absence of calcium (Heilbrunn *et al.*, 1946). It was expected that the exposure time at which increased viscosity occurred would parallel the exposure time necessary for the appearance of parthenogenesis. Accordingly, viscosity determinations were made during the progress of determinations of parthenogenetic activity at the time that samples of eggs were removed to sea water (for a discussion of the method of viscosity determination see above).

TABLE 2

PER CENT OF CLEAVAGE OBTAINED WITH VARIOUS ACIDS AND WITH THE ACID EXTRACTS

Extract	Butyric Acid	Acetic Acid	Lactic Acid	Phosphoric Acid
12.0	6.5	5.0	4.0	.....
11.0	15.0	13.0	7.0	.....
12.0	7.0	6.0	3.0	2.0
14.5	11.0	8.0	1.0	6.0
16.0	4.0	7.0	5.0	.....
28.5	16.0	21.0	3.0	.....
21.0	14.0	5.0	2.0	.....
21.0	20.0	16.0	2.0	.....
19.5	10.0	14.0	0	.....
36.0	28.0	39.0	1.0	.....
16.0	6.0	12.0	0	0
37.0	33.0	34.0	2.0	3.0
54.0	35.0	44.0	6.0	.....
36.0	39.0	36.0	7.0	3.0
32.0	44.0	38.0	14.0	1.0
29.0	30.0	22.0	3.0	1.0
45.0	17.0	31.0	0	0
41.0	30.0	42.0	1.0	1.0
45.0	41.0	49.0	0	.....
15.0	17.0	17.0	0	0
Ave. 27.08	21.18	22.95	3.05	1.7

The results are shown in Figure 9. The viscosity and the per cent of cleavage are plotted against the exposure time on the same graph. The left-hand ordinate shows the time of centrifugation used in determining the viscosity, the right-hand ordinate shows the per cent of cleavage, and the abscissa shows the time of exposure of the eggs to the extract. The expected relationship was not found. Ac-

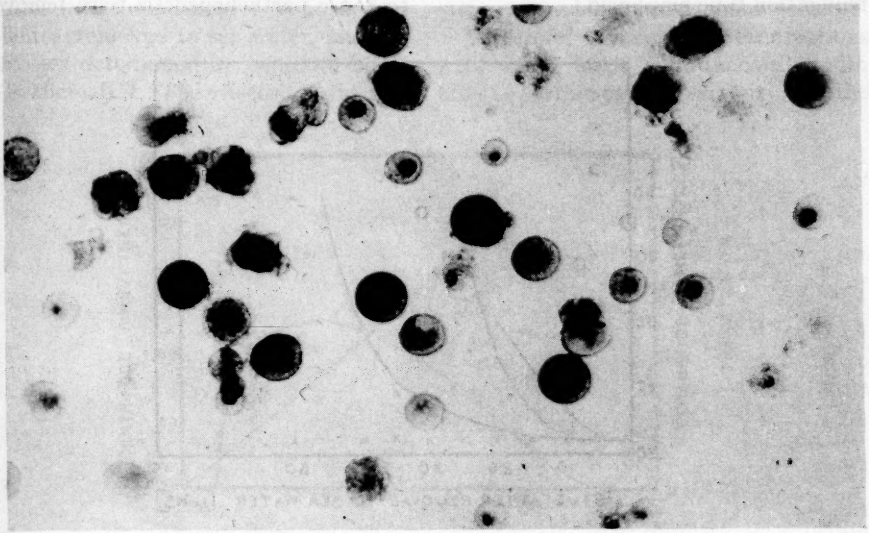

FIG. 8.—Eggs treated with acetic acid. Note cytolysis

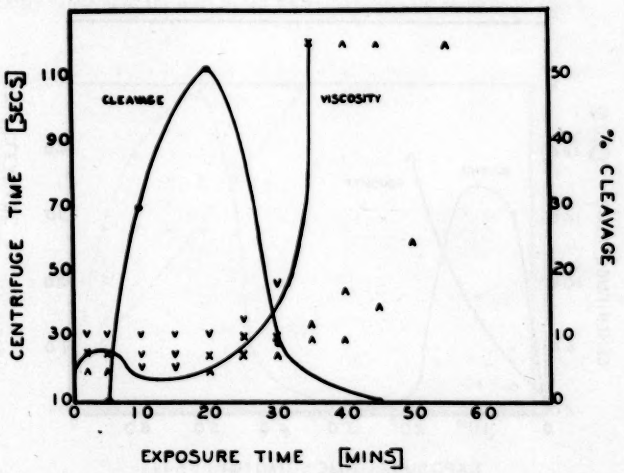

FIG. 9.—Viscosity and per cent of cleavage for various exposure times. Viscosity determinations were made immediately after removal of the eggs to sea water. *V* indicates that the eggs were stratified; *Λ*, that the eggs were not stratified; and *X*, that the eggs were partially stratified.

tually, the viscosity seemed to begin to rise after the parthenogenetic activity had appeared. Eggs that had been exposed for too long a time and consequently showed no cell divisions could not be stratified at all at the highest centrifugation time employed.

It was thought, however, that a reaction might have been initiated which would lead to an increase in viscosity, even after the eggs had been removed to sea water. Thus, if eggs treated with the extract for a length of time just sufficient to cause parthenogenesis were removed

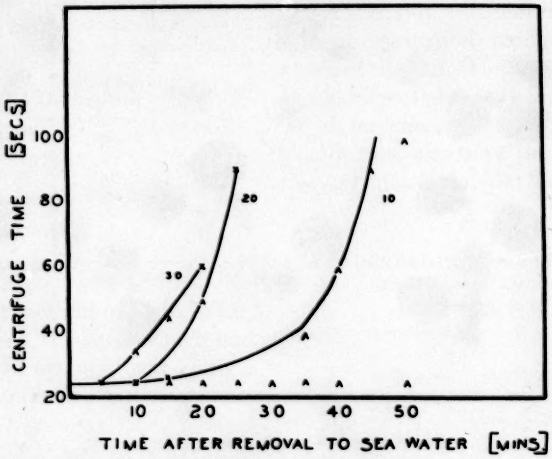

FIG. 10.—Viscosity changes at 5-minute intervals after removal of the eggs to sea water, for eggs exposed to the extract for 10, 20, and 30 minutes. Symbols the same as in Fig. 9.

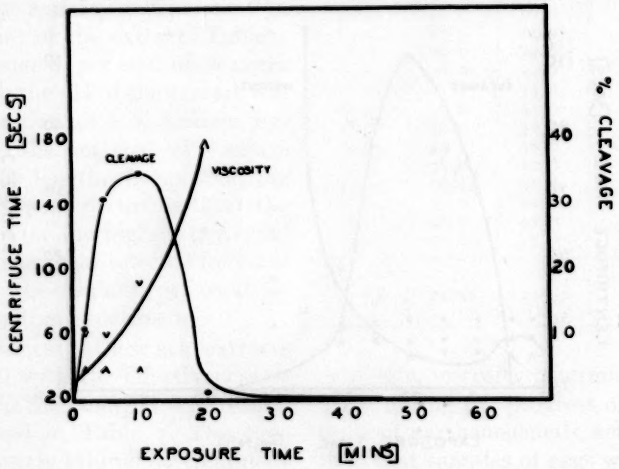

FIG. 11.—Viscosity and per cent of cleavage for various exposure times. Viscosity determinations were made 25 minutes after removal of the eggs to sea water. Symbols the same as in Fig. 9.

to sea water and allowed to stand for a period of time before determination of the viscosity, the reaction initiated by the extract might continue to go on, with an increase in viscosity as a final result. This was found to be the case. Eggs were treated with the extract for 10, 20, and 30 minutes, removed to sea water, and the viscosity determined at 5-minute intervals thereafter. The viscosity did rise after removal of the eggs to sea water, as can be seen from Figure 10. Thus, for example, in the eggs treated with the extract for 10 minutes (curve on the right), a rise could be detected 15 minutes after removal to sea water. By 50 minutes the viscosity had increased about fourfold.

Therefore, viscosity determinations were again made simultaneously with tests of parthenogenetic activity, but the

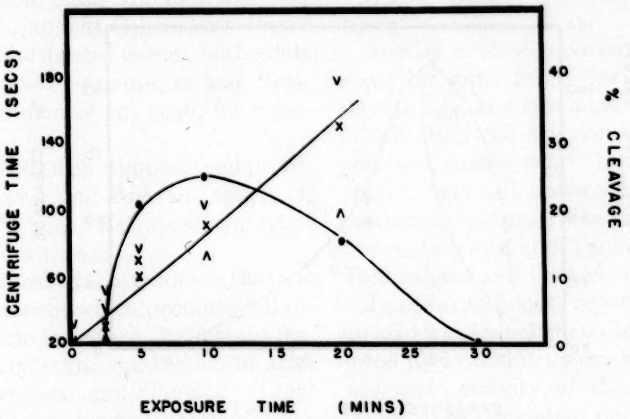

FIG. 12.—Viscosity and per cent of cleavage for various exposure times. Viscosity determinations were made 50 minutes after removal of the eggs to sea water. Symbols the same as in Fig. 9.

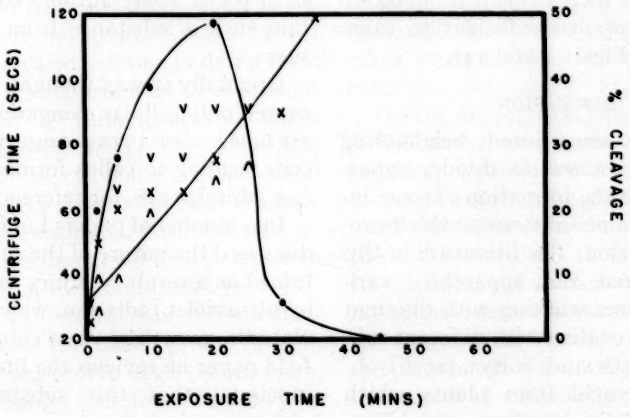

FIG. 13.—Viscosity and per cent of cleavage for various times of exposure to butyric acid. Viscosity determinations were made immediately after removal of the eggs to sea water. Symbols the same as in Fig. 9.

measurements were made 25 and 50 minutes after removal of the eggs to sea water. In this way an increase in viscosity could be detected after exposures which produced the first signs of divisions (Figs. 11 and 12).

Butyric and acetic acids also cause an increase in viscosity, but this increase occurs rapidly. Even when determinations of viscosity are made at the time of removal of the eggs from these acids to sea water, an increase can be detected after exposures just sufficient to cause cell division (Figs. 13 and 14).

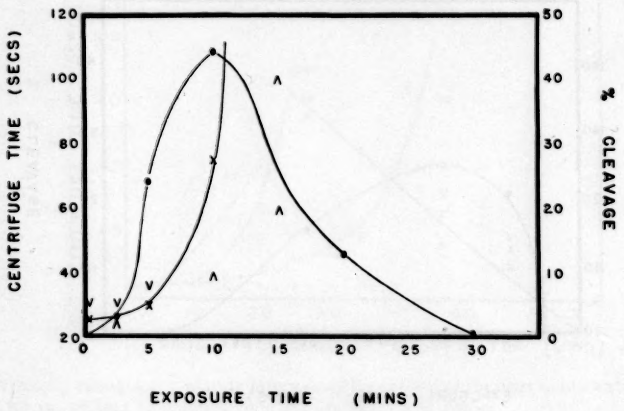

FIG. 14.—Viscosity and per cent of cleavage for various times of exposure to acetic acid. Viscosity determinations were made immediately after removal of the eggs to sea water. Symbols the same as in Fig. 9.

DISCUSSION

When cells are injured, neighboring cells may be caused to divide, apparently owing to the formation of some injury substance or substances which promote cell division. The literature in this field is scattered, and, apparently, various investigators working with different organisms are dealing with different substances. Umrath and Soltys (1936) obtained an oxyacid from plants which stimulated cell division. Bonner and English (1937, 1938) claim to have isolated and purified a wound hormone from plants, which they call "traumatin." This is apparently a dicarboxylic acid, 1-decene-1,10-dicarboxylic acid, and is soluble in water and alcohol (English, Bonner, and Haagen-Smit, 1939*a*, *b*, and *c*). Its activity is dependent on the two carboxyl groups and is increased by the presence of the double bond. "Traumatic acid" is potent in stimulating the growth of intumescences on the inner surface of bean pods. These authors could not obtain such a substance from animal tissues.

Originally it was thought that auxins caused only cellular elongation, but they are now known to cause multiplication of cells, leading to callus formation as well (see Morel, 1948, for references).

In a number of papers Loofbourow has discussed the nature of the substance obtained as a result of injury of yeast cells by ultraviolet radiation, which can stimulate the growth of yeast cultures. In his 1948 paper he reviews the literature and concludes that this substance is an adenine nucleotide.

Substances which will induce cell divi-

sion have been isolated from animal tissues as well. Various workers have succeeded in extracting substances from human tissues which will induce malignant tumors in mice (Neufach, 1937; Schabad, 1937*a*, *b*, 1945; Des Ligneris, 1940; Hieger, 1940, 1941, 1946, 1947; Kleinenberg *et al.*, 1940, 1941; Menke, 1940, 1942; Steiner, 1940, 1941, 1942, 1943; Wachtel, 1946; and Steiner *et al.*, 1947). These substances are obtained from the nonsaponifiable lipid fraction and are thought to be sterols. Others have found that extracts of animal tissues, both adult and embryonic, promote wound healing (see Hoffman *et al.*, 1946, for references).

Nucleic acids also stimulate cell proliferation (Shaver and Brachet, 1949); cf. also Fischer, 1941; Astrup *et al.*, 1947; and Waymouth, 1947).

There is considerable evidence that the injury substances which promote cell division are acid in nature. A constant feature of injury is the production of acid. Thus Chambers and Pollack (1927) found by the use of indicators that injury to a cell causes a drop in pH of an entire unit or more. If the cytoplasm is torn slowly, this reaction is slight, indicating that a rapid neutralization occurs if the injury is not too great. Crozier (1918) also found an acid reaction in dying cells; Moore (1917) reports acid development as the result of injury to nervous tissue in frogs; Bowers (1943) mentions acid production in wounded areas; and Fischer (1933) reports an acid reaction around tissue explants. Pathological conditions are accompanied by a decrease in pH, according to Schmidtman (1925).

Neither iodoacetate nor sodium fluoride inhibits the acid production in cytolyzing sea-urchin eggs (Runnström, 1935). Probably, therefore, the acid produced is not a product of the usual type of glycolysis. Phlorizin does inhibit this acid formation (Rothschild, 1939; see also Cennamo and Montella, 1947, who believe that a breakdown of ATP with production of phosphoric acid is responsible). It has been suggested that this acid may be the product of an oxidative decarboxylation of carbohydrates (Lindberg, 1946).

Acid is also produced when sea-urchin eggs are fertilized or activated by parthenogenetic agents. Runnström (1935) believes this to be identical with the acid of injury.

Acid is a very good parthenogenetic agent for some forms, for example, the starfish egg, and the importance of acid in initiating cell division has been emphasized many times by R. S. Lillie (1941). Acid soil, moreover, leads to the production of tumor-like growths in cotton roots (Gore and Taubenhaus, 1931; Taubenhaus and Ezekiel, 1931).

In the experiments reported here, also, an acid was found when the cells were injured by heat. Furthermore, the parthenogenetic activity of the extracts was found to be dependent on their acid nature, since neutralization destroyed the effectiveness. It is obvious from these experiments that the active acid is neither phosphoric acid nor lactic acid, since these acids have essentially no activating effect when used at the same pH as the extracts.

Hultin (1949, 1950) has recently reported that homogenates of sea-urchin eggs do not produce acid in the presence of citrate but that the pH drops suddenly on the addition of calcium. According to the colloid-chemical theory of cell division, a release of calcium by the activating agent is essential to reactions causing the increase in viscosity necessary for cell division. It is not unlikely, therefore, that the acid produced on activation is dependent on a release of calcium.

The acid extracts from injured tissues

reported here also cause an increase in protoplasmic viscosity. In view of the idea that viscosity changes are of prime importance in cell division, it is interesting that injury to cells causes an increase in viscosity, as well as mitosis in neighboring cells. Thus Bünning (1926) found that the viscosity increases at least three and a half to six times in cells near a wounded surface in *Secale* and *Raphanus*. Kostoff (1930*a*, *b*) and Kostoff and Kendall (1930) found an increase in viscosity as far as 2 mm. away from a cut surface, as well as a high viscosity in gall tissues and in tumors of *Nicotiana* hybrids. He says that the rapid proliferation of the tumors appears to be due to the favorable conditions created by the increased cytoplasmic viscosity.

Because blood coagulation requires the presence of calcium and because increased viscosity represents a clotting of the protoplasm, it has been suggested that the increase in viscosity preceding the appearance of the mitotic spindle is due to a thrombin-like substance (Heilbrunn and Wilson, 1949). Other workers have shown the importance of clotting substances in cell division. Fischer (1933) was led to suggest that thrombin or prothrombin may play an important part in cell division because of the following facts: (1) If a wounded area in a tissue culture is washed thoroughly to remove growth-promoting substances released as a result of injury, the wound does not heal. Moreover, Tyrode solution removed from a wounded area contains substances which accelerate coagulation of chicken plasma. Thus diluted chicken plasma clotted in 200 minutes when Tyrode solution was added from an unwounded culture which had stopped growing and in 2 minutes when Tyrode solution, taken 10 minutes after injury, was added (Fischer, 1930). Other workers have reported release of clotting substances following injury (Pollard and Stoker, 1946; Stoner and Green, 1947; Moreau *et al.*, 1948). (2) Embryo extracts which stimulate growth of tissue cultures also cause a pronounced acceleration of clotting (Fischer, 1930). The effect of embryo extracts on coagulation time was confirmed by Jacoby (1937), who also found that a dialyzate of embryo juice hastens coagulation. (3) A wound in a tissue culture does not heal if it is bathed in a heparin solution. The heparin is not toxic to the cells. The inhibition of growth and of coagulation by heparin both disappear after a short heating with hydrochloric acid, which does not cause a breakdown of the molecule, according to Fischer (1936), but a molecular rearrangement.

Zakrzewski (1932*a*, *b*, *c*; 1933) also believes that prothrombin plays an important role in cell division. He found that prothrombin stimulates growth of tissue cultures in Tyrode solution and that heparin will prevent this stimulating action of prothrombin. He suggests that, *in vivo* as well as *in vitro* (in tissue culture), cell proliferation is probably caused by the liberation of prothrombin and is controlled by the alternate action of prothrombin and antiprothrombin (heparin).

Moreover, the activating effect of injury on frogs' eggs is greatly enhanced by the introduction of small amounts of blood or blood serum, as well as cell-free water extracts of testes and blood (Einsele, 1930).

Further evidence for the theory that clotting substances are involved in cell division has come from work in this laboratory. Heparin inhibits cell division in the fertilized *Chaetopterus* egg and, at the same time, prevents the increase in viscosity preceding the appearance of the mitotic spindle (Heilbrunn and Wilson, 1949). Heparin also inhibits, to some extent, the activation of frogs' eggs caused

by pricking in the presence of blood (Harding, 1949). The bacterial polysaccharide of Shear is a heparin or a heparin-like substance. It gives a metachromatic reaction with toluidine blue and delays blood clotting (Heilbrunn and Wilson, 1950). It also inhibits division in the *Chaetopterus* egg (Heilbrunn and Wilson, 1950) and activation of the frog egg by blood (Harding, unpublished results). Its effectiveness in causing regression of tumors in mice (Shear *et al.*, 1944) is of interest in the light of this theory.

Since acid formation and thrombin formation are both dependent on the presence of calcium and since calcium has been shown to be important in cell division (Heilbrunn and Young, 1930; Heilbrunn and Wilbur, 1937), it is suggested that the acid formed by injury bears some relation to the clotting of protoplasm leading to cell division.

SUMMARY

1. Extracts of injured tissues cause artificial parthenogenesis in the egg of *A. punctulata*.

2. These extracts are acid. Neutralization of them causes loss of parthenogenetic activity.

3. The effectiveness of the extracts was compared with the effectiveness of other acids at the same pH. The averages of the highest per cent of cleavage from each experiment were as follows: for the extract, 27.08; for butyric acid, 21.18; for acetic acid, 22.95; for lactic acid, 3.05; and for phosphoric acid, 1.7.

4. After exposures just long enough to cause artificial parthenogenesis, an increase in protoplasmic viscosity can be detected.

5. It is concluded from these results that an acid substance is released as the result of injury and that this acid can cause an increase in protoplasmic viscosity and initiate cell division in the *Arbacia* egg.

6. These results are discussed in relation to the colloid-chemical theory of cell division.

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STUDIES ON THE INORGANIC REQUIREMENTS OF TETRAHYMENA[1]

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THE organic nutrients required by *Tetrahymena geleii* have been the subject of numerous investigations, and a considerable body of information has accumulated (Kidder and Dewey, 1948*a*, 1949*a*, *b*; Dewey, Parks, and Kidder, 1950). The mineral requirements for this animal microörganism are still largely unknown. This lack of information is due principally to the fact that both the organic and the inorganic constituents of the complex medium used invariably contain contaminating minerals

[1] Aided by a grant from Research Corporation and a grant recommended by the Committee on Growth acting for the American Cancer Society.

in varying concentrations. These contaminating ions make it difficult indeed to determine exact quantitative requirements, and, in the case of elements required only in traces, even qualitative requirements are not detected.

While it has been the practice to include inorganic mixtures ("physiological salts") in compounding media for the growth of *Tetrahymena* (Dewey, Kidder, and Parks, 1950), very little has been attempted on the question of exact inorganic requirements. Hall and Cosgrove (1945) studied this question in a crude medium, and Kidder and Dewey (1949*a*) reported an increased copper and iron requirement in certain types of media.

The present report deals with investigations on the mineral requirement of *T. geleii* W and demonstrates that, in the complex media which must be used, phosphate, magnesium, and potassium represent definite requirements, while none could be shown for calcium, manganese, and sulphate. The same is true of the trace elements, for technical reasons.

MATERIAL AND METHODS

Tetrahymena geleii strain W was used throughout this investigation. The organisms were grown in pure (bacteria-free) culture. The basal medium was Medium A of Dewey *et al.* (1950) (Table 1), except that in some cases (see Fig. 2) Tween 80 was substituted for Tween 85 at the level of 10 mg/ml of medium. When phosphate was being studied, the nucleotides were omitted, guanosine was added at 50 γ/ml to meet the purine requirement, and the level of uracil was raised to 20 γ/ml. Appropriate omissions of ions were made in the various experiments. Thus, when potassium was under investigation, sodium phosphate was

TABLE 1

COMPOSITION OF BASAL MEDIUM*

DL-alanine	110	Thiamine HCl	1.00
L-arginine	206	Biotin (free acid)	0.0005
L-aspartic acid	122	Choline Cl	1.00
Glycine	10		
L-glutamic acid	233	$MgSO_4 \cdot 7H_2O$	100†
L-histidine	87	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
DL-isoleucine	276	$MnCl_2 \cdot 4H_2O$	0.5
L-leucine	344	$ZnCl_2$	0.05
L-lysine	272	$CaCl_2 \cdot 2H_2O$	50
DL-methionine	248	$CuCl_2 \cdot 2H_2O$	5
L-phenylalanine	160	$FeCl_3 \cdot 6H_2O$	1.25
L-proline	250	K_2HPO_4	1,000
DL-serine	394	KH_2PO_4	1,000
DL-threonine	326		
L-tryptophane	72	Guanylic acid	30‡
DL-valine	162	Adenylic acid	20
		Cytidylic acid	25
Ca pantothenate	0.10	Uracil	10
Nicotinamide	0.10		
Pyridoxine HCl	1.00	Dextrose	2,500
Pyridoxal HCl	0.10	Na acetate	1,000
Pyridoxamine HCl	0.10	Tween 85	700
Riboflavin	0.10		
Pteroylglutamic acid	0.01	Protogen§	1 unit

* All values, except for that of protogen, in γ/ml of final medium.

† Individual salts omitted in appropriate experiments.

‡ In all experiments involving phosphate requirement, nucleosides were used instead of the nucleotides.

§ Protogen obtained from the Lederle Laboratories through the courtesy of Dr. E. L. R. Stokstad.

substituted for potassium phosphate; and when phosphate was being studied, potassium chloride was included to insure against potassium limitations.

All experiments were run in triplicate and were repeated varying numbers of times. In certain cases, in order to make sure that the organisms were depleted with respect to a certain ion, three or more serial transplants were carried out. Incubation was for 96 hours at 25° C., with the tubes in a slanted position for maximum aeration (Kidder and Dewey, 1948*b*), and growth responses were measured turbidimetrically.

The loading and inoculating of experimental series (except where serial transplants were being carried out) were accomplished by the use of the Brewer automatic pipetting machine. Since our method has not been described before, it will be briefly outlined here.

In our experimental work with *Tetrahymena* the organisms are grown in 15 × 125-mm. test tubes in 4 ml. of medium. All experiments are run in triplicate. A distilled-water blank and a tenfold dilution of the variable are used in each dose-response test. Thus eleven rows of three tubes each are employed in every test.

The variable and the water are added to the tubes, by means of the Brewer automatic pipetting machine, fitted with a 3-ml. Luer-Lok syringe calibrated to deliver 0.2 ml. or 1.0 ml., depending upon the cam setting. A No. 17 2-inch hypodermic needle is used as a delivery tip. The Brewer is first adjusted to deliver 0.2 ml., and the system is filled with a solution of the variable at double the highest concentration desired. Employing the principle of multiple deliveries per tube, rows 2–5 receive 0.2, 0.4, 0.6, and 0.8 ml., respectively. Rows 7–10 are loaded in an identical manner. The cam setting is now adjusted so that the machine will deliver 1.0 ml., and this amount is delivered into rows 6–10. Row 11 receives 2.0 ml. (two deliveries per tube). Thus a graded spread of variable from 0.2 to 2.0 ml. in steps of 0.2 ml. is achieved. The valve assembly of the machine is now rinsed thoroughly with tap water, followed by distilled water, and the above procedure is performed in reverse order (starting with row 10) with distilled water. This brings the volume to 2.0 ml. in each tube. The tubes are then covered with aluminum caps and sterilized by autoclaving. We have found the aluminum caps superior to cotton plugs in the ease and speed with which they may be handled. Where heat-labile compounds are to be used as the variable, sterile filtered solutions may be added aseptically by the foregoing procedure to the sterile tubes containing appropriate amounts of water.

The basal medium minus the variable, or with other desired modifications, is made up at double the final concentration. This is placed in an Erlenmeyer flask equipped with a cotton-plugged thistle tube and a bent-glass outlet tube. The neck of the flask and the tip of the outlet tube are covered, as shown in Figure 1. The flask is autoclaved with the outlet tube raised above the surface of the fluid, to avoid expulsion of the medium through the tube during autoclaving.

The valve assembly and rubber tubing are detachable as a unit from the rest of the pipetting machine. This, together with the attached delivery tip (No. 15 2-inch hypodermic needle) and a 10-ml. Luer-Lok syringe, are wrapped and sterilized.

Upon cooling, the entire flask of medium is inoculated (Stokstad *et al.*, 1949) by adding the desired volume of a 4–7-day-old culture of *Tetrahymena* through the thistle tube (Fig 1). We have found a 1:200 inoculum satisfactory, i.e., 1 ml. of inoculum per 200 ml. of single-strength medium. Other substances for which sep-

arate autoclaving is desirable may also be added through the thistle tube. Dextrose caramelization is avoided by adding the dextrose to the base aseptically after autoclaving.

The glass outlet tube is connected to the rubber intake tube, using aseptic precautions, and the flask is clamped in an inclined position on the shaker. The shaker is employed to insure an even distribution of organisms during tube inoculation. The Brewer is adjusted to deliver exactly 2.0 ml., and the system is filled. The cap is removed from the first tube and set aside, and the tube is loaded. While the first tube is being loaded, the cap is removed from the second tube and is placed on the first when loading is completed. The second tube is now filled, and the cap from the third tube is placed on it. This procedure is continued until all tubes are filled. The cap set aside from the first tube is now placed on the last tube. This procedure is readily mastered with a little practice. A rate of 30–40 tubes loaded per minute is the usual speed employed.

FIG. 1.—Illustration of the setup used for the aseptic automatic dispensing of inoculated double-strength basal medium into tubes containing previously sterilized variable and water.

RESULTS

Sodium and chloride.—For technical reasons, no attempt has been made to investigate the importance of sodium or chloride in the metabolism of *Tetrahymena*.

Calcium and magnesium.—The omission of all calcium salts (with the exception of calcium pantothenate) from the medium does not lower the growth of this organism. In attempts to remove traces of calcium which might be found present as contaminants, varying concentrations of citrate and oxalate were added to the medium. It is known that these compounds readily form complexes with both calcium and magnesium (MacLeod and Snell, 1947). Both citrate and oxalate in-

hibited growth, and this growth inhibition could be partially overcome by adding high levels of calcium salts. However, inhibition could be completely overcome

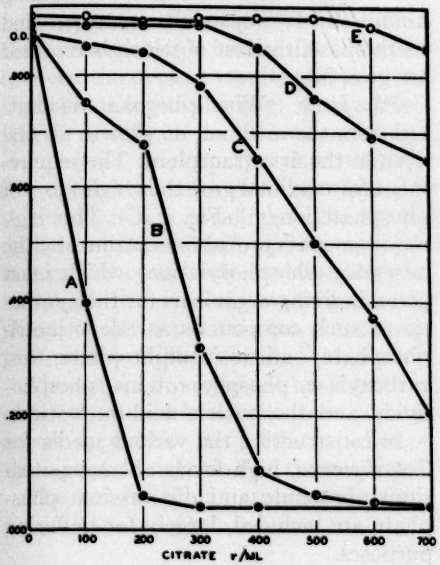

FIG. 2.—Response of *T. geleii* W to sodium citrate in basal medium *A* minus magnesium salts and containing 10 mg/ml Tween 80 instead of Tween 85. The amounts of $MgSO_4 \cdot 7H_2O$ (in γ/ml) are as follows: *A* = 25; B = 50; *C* = 100; *D* = 150; *E* = 200. Growth recorded as optical density where 0.000 is obtained with distilled water.

by raising the magnesium content of the medium (Fig. 2). In the presence of high citrate or oxalate and high magnesium it can be calculated that essentially all the calcium was removed and optimum growth still resulted. It must be concluded, therefore, that calcium does not represent an absolute requirement for *T. geleii* W, while magnesium is indispensable. The omission of magnesium from the medium (without citrate or oxalate additions) results in nearly complete absence of growth in the first transplant. Linear growth occurs in response to increasing concentrations of magnesium (Fig. 3, *A*), and half-maximal growth is obtained upon the addition of 2 γ of Mg^{++}/ml (as $MgSO_4 \cdot 7H_2O$).

Manganese ions failed to effect the release of inhibition caused by citrate additions and did not increase the effectiveness of magnesium. This is in contrast to the results obtained with certain lactic acid bacteria by MacLeod and Snell (1947).

Potassium.—When all potassium salts are omitted from the medium, the growth is reduced to less than one-fourth of normal (Fig. 3, *B*). The addition of potassium (in the form of potassium chloride) restores growth, and under

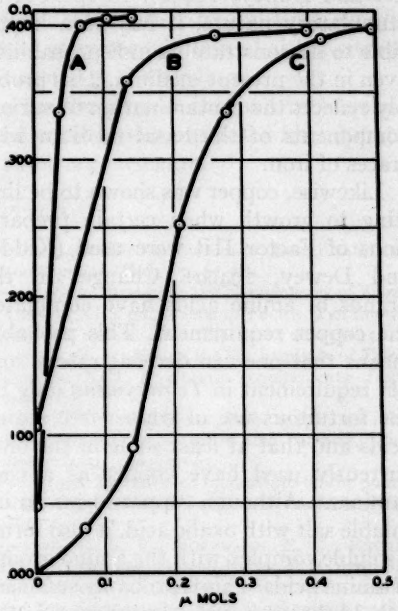

FIG. 3.—Dose response of *T. geleii* W to increasing amounts of Mg^{++} (curve *A*); K^+ (curve *B*); PO_4^{---} (curve *C*), plotted on a molar basis.

these conditions 5 γ of K^+/ml of medium allows half-maximal growth, and 20 γ/ml allows maximal growth. These values should not be considered absolute, however. The probability of potassium con-

tamination of constituents of the medium is to be inferred from the residual growth (even in serial transplants) of the organism when known sources of potassium were withheld. Also it must be remembered that the level of sodium present may influence greatly the quantitative requirement of cells for potassium (MacLeod and Snell, 1948), and the present medium does contain appreciable amounts of sodium brought in during neutralization.

Iron and copper.—Iron was earlier found to increase the growth of *T. geleii* W when added to a medium lacking what was formerly called "Factor IIB" (Kidder and Dewey, 1949*a*). In spite of its stimulatory nature, it has been impossible to demonstrate its indispensability, even in the present medium. This probably reflects the contamination of various components of the basal medium with traces of iron.

Likewise, copper was shown to be limiting to growth when certain preparations of Factor IIB were used (Kidder and Dewey, 1949*a*). Changes in the brands of amino acids have eliminated the copper requirement. This probably means that one can demonstrate a copper requirement in *Tetrahymena* only by the fortuitous use of copper-free amino acids and that at least some of the ones currently used have copper as a contaminant. Although copper forms an insoluble salt with oxalic acid, it also forms a soluble complex with the amino groups of amino acids. This is probably sufficient to hold the copper in solution even in the presence of oxalate.

Trace elements.—No demonstration of a requirement for manganese, zinc, cobalt, fluorine, borate, or molybdate has been possible so far. Again it seems probable that contaminations bring in adequate amounts of those trace elements needed by the organism.

Sulphate.—It has been impossible to demonstrate a sulphate requirement. This may simply mean that such a requirement can be met by the oxidation of the sulphur of the sulphur-containing amino acids (cysteine, cystine, and methionine, the last of which is required for growth).

Phosphate.—When phosphate is omitted from the medium, no growth occurs, even in the first transplant. The requirement for maximal growth is high (50 γ of phosphate ion/ml, Fig. 3, *C*). This high requirement is probably explained by the necessary phosphorylations which must go on in living organisms for the synthesis of such compounds as adenosinetriphosphate, adenosinediphosphate, cocarboxylase, phosphoproteins, phospholipids, and the nucleic acid nucleotides.

In constructing the various media for *Tetrahymena*, high levels of monopotassium phosphate and dipotassium phosphate are included, largely for buffering purposes.

DISCUSSION

The presence of such chelating agents as amino acids, Tween, etc., complicates the picture of inorganic requirements. It is possible that, in spite of the presence of oxalate in the medium sufficient to precipitate all the calcium ions present, enough remains dissolved as complexes to supply the requirements. Unless the requirement is minute enough to be satisfied by trace contaminations of other components of the medium, the fact that the omission of calcium from the medium does not reduce growth would seem to indicate lack of a requirement. When the increased calcium necessary to give half-maximal growth is plotted against the citrate concentration, as suggested by Hutner *et al.* (1950), the curve does not have an intercept on the axis for zero concentration of citrate, and therefore no

extrapolation can be made to determine the calcium requirement. However, the situation is complicated by the existence of a high magnesium requirement, which could obscure a small calcium one.

It is possible that the effect discussed above is responsible for the discrepancies between the present results and those of Hall and Cosgrove (1945) on *T. geleii* GP. It is difficult to understand, however, how a casein preparation entirely free from calcium could be obtained, since milk is rich in calcium. The results of these authors with phosphate and with iron can be completely explained by the nature of their medium, since casein is a phosphoprotein and milk is a notoriously poor source of iron.

The approach suggested by Hutner *et al.* (1950) should be a fruitful one and deserves application to further studies of the inorganic nutrition of these ciliates.

The results of our experiments seem important, in that they demonstrate some of the inorganic requirements of an animal cell and also show how difficult it is to determine trace-element requirements, probably because of contaminations of materials used in the medium. Definitive experiments regarding trace-element requirements must await more highly purified chemicals.

SUMMARY

1. By omission experiments it has been found that *T. geleii* W possesses a requirement for potassium, magnesium, and phosphate.

2. No requirement for calcium could be demonstrated either by omission experiments or by the addition of either citrate or oxalate to the medium.

3. In the present medium no requirement could be demonstrated for sulphate or the trace elements.

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THE CONTROL OF RECONSTITUTIONAL DEVELOPMENT IN PLANARIANS WITH PILOCARPINE

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THE action of pilocarpine on developmental processes is of much interest but is not entirely understood. Wolsky, Tazelaar, and Huxley (1936) showed that when frog's eggs were allowed to develop in dilute solutions, the head of the tadpole was differentially increased in size. They attributed this condition to differential acceleration. Rulon (1941) found that cleavage in sand-dollar eggs was stimulated by low concentrations of the agent but that, if the exposure was prolonged, the effect became definitely inhibitory. Continuous exposure to dilute solutions gave plutei with enlarged oral lobes. This condition was somewhat similar to that found with frog tadpoles but was attributed to differential conditioning rather than to differential acceleration. In experiments with a gradient of concentration across sand-dollar eggs, Pease (1942) found that pilocarpine appeared to act specifically on bilateral determination without differential cleavage inhibition.

In our general research program, which deals with the factors in developmental patterns and their possible control, it seemed of interest to test the action of pilocarpine on reconstitutional processes in planarians.

MATERIAL AND METHODS

The planarians (*Dugesia dorotocephala*) were collected in large numbers from a spring-fed stream in a quarry near Cary, Illinois. In the laboratory the stock was kept in large covered pans on a water table. It was fed on beef liver twice a week, and the water (Evanston tap water, aerated at least 24 hours before using) was changed regularly. Only animals measuring 14–16 mm. in length and starved for at least 1 week were used in these experiments. Each test involved pieces from 50 animals. A tap-water control with the same number was always run at the same time. In sectioning small pieces it was difficult to cut all exactly the same size. In order to decrease this source of error, 65 animals were sectioned, 50 pieces of most nearly the same size being selected for each lot. After sectioning, the test and control pieces were placed in covered finger bowls in approximately 150 cc. of solution for approximately 10 days. At the end of this time the reconstituted pieces were examined under a microscope and classified in the following manner:

A. Head types[1] and tail regeneration
- Ia. Normal or enlarged triangular heads with auricles at lateral margins and two distinct eyespots; tails inhibited or absent
- Ib. Normal or enlarged triangular heads with auricles at lateral margins and two distinct eyespots; normal tails
- II. Teratophthalmic; triangular heads with auricles at lateral margins but with eyespots showing all degrees of approximation to the median plane, from two distinct eyespots with pigment connected, up to complete cyclopia; normal tails

[1] See Child (1941, pp. 177–81) for figures and full description of head types.

III. Teratomorphic; head more or less rounded in outline, with auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle and with a single, or apparently single, median eye; normal tails
IV. Anophthalmic; head rudimentary, without eyes and with or without a single median auricle; normal tails
V. Acephalic; head completely absent; normal tails

B. Apolar; heads and tails completely absent
C. Bipolar; head regeneration at both the anterior and posterior cut surfaces
D. Dead; pieces dying during the course of the experiment

In a number of cases the head-frequency index (Child, 1941, p. 745) was calculated. This measurement is an approximate "average" for degree of head regeneration for lots of pieces from any particular level. It is arrived at by giving to each head type a number which approximates its degree of inhibition (normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20). The actual number of each head type for any particular level is then multiplied by the number corresponding to its degree of inhibition, and the sum of all is divided by the total number of animals reconstituting from that level.

EXPERIMENTAL

I. EFFECT OF PILOCARPINE ON HEAD FREQUENCY

The animals were sectioned as shown in Figure 1, with the head and posterior half being discarded.[2] Fifty pieces (each approximately one-eighth the length of the entire animal) from the various levels (*A*, *B*, *C*, *D*) were placed in different concentrations of pilocarpine for varying intervals of time. Equal numbers of similar pieces were always allowed to reconstitute in aerated tap water as controls.

After a series of preliminary experiments it was determined that M/1,000 pilocarpine was just below the lethal level; that is, whole animals or pieces could usually tolerate this concentration for 10–14 days without cytolyzing. Accordingly, in one of the first experiments,

1

FIG. 1.—Levels *A*, *B*, *C*, and *D* used in head-frequency experiments. Each level is approximately one-eighth the length of the animal, exclusive of the head.

the pieces were allowed to reconstitute in this concentration. In Table 1 are recorded the percentages of different head types that emerged from pieces exposed to M/1,000 pilocarpine, as well as from the tap-water control. It will be noted that this concentration of pilocarpine induced the development of a higher percentage of normal or near-normal heads, particularly at the posterior levels. In Table 1 are also recorded the head-frequency indices of tests and controls. Comparison of these data gives a summary of the degree of head formation in test and control lots.

It seemed of interest to compare the effects of pilocarpine at different concen-

[2] The head was discarded because it has little capacity for reconstitution. The posterior region was not used because it includes the zones of fission of posterior zoöids. This particular study on head frequency was therefore limited to a well-defined region of the anterior zoöid (i.e., between mouth and head).

trations and exposure periods. Six experiments were therefore conducted with solutions of pilocarpine ranging from M/15,000 for the entire reconstitutional period to M/500 for the first 30 hours after section. The data from these experiments were analyzed for head frequency, and in Table 2 are recorded the differences in the head-frequency indices of the pilocarpine tests and the paired tap-water controls. It will be noted that in every experiment and at every level the tests gave a higher head frequency than did the controls. It should be pointed out, however, that in head-frequency experiments the data from posterior levels hold the greatest significance, for these levels under normal conditions give few normal heads and commonly an entire lot will develop only into headless (acephalic) individuals. In Table 2 it may also be noted that a short time exposure (30–48 hours) to the highest concentrations (M/500) was slightly more effective in increasing head frequencies.

Since pilocarpine proved so effective in increasing head frequency when the pieces were treated, it was decided to test the effect when the intact parent-animals were exposed to the agent for some time, then sectioned, and the pieces

TABLE 1

HEAD FREQUENCY AND VIABILITY OF TRANSVERSE PIECES OF *D. dorotocephala* WITH CONTINUOUS EXPOSURE TO PILOCARPINE NITRATE
(Head Types and Deaths Given in Percentages)

Head Types and Measurements	Aerated Tap-Water Control				M/1,000 Pilocarpine			
	A	B	C	D	A	B	C	D
Normal	96	42	20	2	96	78	34	22
Teratophthalmic	0	28	14	4	4	20	30	20
Teratomorphic	2	4	8	6	0	0	14	4
Anophthalmic	2	14	32	16	0	0	22	38
Acephalic	0	12	24	68	0	0	0	16
Dead	0	0	2	4	0	2	0	0
Head-frequency index	98	75	55	30	99	96	75	59
Increase in head frequency (indices of test minus control)	.....	.....	.....	.....	+1	+21	+20	+29

TABLE 2

DIFFERENCE IN THE HEAD FREQUENCIES OF TESTS AND CONTROLS IN *D. dorotocephala* WITH VARIOUS EXPOSURES OF THE TESTS TO PILOCARPINE NITRATE

Pilocarpine Solutions and Exposure Periods	Increase (+) or Decrease (−) in Head-Frequency Indices of Test to Control			
	A	B	C	D
1. M/15,000, entire reconstitution period	+6	+10	+22	+27
2. M/5,000, entire reconstitution period	+2	+10	+18	+35
3. M/2,500, entire reconstitution period	+6	+ 8	+20	+27
4. M/2,500, first 26 hours of reconstitution period	+1	+12	+17	+30
5. M/500, first 48 hours of reconstitution period	+2	+22	+33	+38
6. M/500, first 30 hours of reconstitution period	+1	+22	+21	+37

Since pilocarpine proved so effective in increasing head frequency when the pieces were treated, it was decided to test the effect when the intact parent-animals were exposed to the agent for some time, then sectioned, and the pieces

allowed to reconstitute in tap water. When the uncut animals were first placed in the test solutions, their muscular activities were greatly increased, but, by the time of sectioning, movement had usually returned to normal. In one experiment (Table 3) the parent-animals were exposed to M/1,000 pilocarpine for 5 days. When returned to tap water and sectioned, the pieces from all levels showed a *decrease* in head frequency. In a second experiment the intact animals were exposed for only 48 hours to M/500 pilocarpine. They showed, on reconstitution of the pieces in tap water, a decrease in head frequency at the *A*, *C*, and *D* levels but a slight increase at the *B* level. These data show that pilocarpine before section acted quite differently from the way it did with exposure beginning after section. They indicate that conditions in the parent-animal may be so altered by the agent that the normal reconstitutional capacities of the pieces may not be realized.

TABLE 3

DIFFERENCE IN THE HEAD FREQUENCIES OF TESTS AND CONTROLS IN *D. dorotocephala* FOLLOWING EXPOSURE OF THE TEST ANIMALS TO PILOCARPINE BEFORE SECTIONING

PILOCARPINE SOLUTIONS AND EXPOSURE PERIODS	INCREASE (+) OR DECREASE (−) IN HEAD-FREQUENCY INDICES OF TEST TO CONTROL			
	A	B	C	D
1. M/1,000, 5 days before sectioning	−4	−5	−10	−13
2. M/500, 48 hours before sectioning	−1	+4	−18	− 6

II. EFFECTS OF PILOCARPINE ON SCALE OF ORGANIZATION

Fifty pieces (approximately one-sixteenth the length of the whole animal) were removed from both *A* and *B* levels (Fig. 2) and placed in aerated tap water as controls. Identical pieces and numbers were placed in M/1,000 pilocarpine. After 30 hours the pilocarpine solution was changed for tap water because cytolysis was beginning in some of the pieces. The data are recorded in Table 4. It will be noted that pilocarpine caused a considerable *increase* in forms with normal (or enlarged) heads and inhibited tails (I*a*) at both *A* and *B* levels. There was also an increase in head frequency.

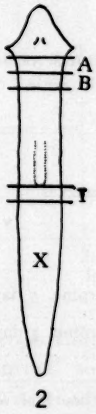

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FIG. 2.—Levels *A* and *B* used in scale of organization experiments, and level *I* used in experiments involving delay of posterior section. Each level is approximately one-sixteenth the length of the animal, exclusive of the head. Posterior halves (seven-sixteenths) used in experiments on rate of head formation designated as *X*.

A second experiment was performed in which the whole animals were exposed to M/1,000 for 5 days, after which *A* and *B* pieces (Fig. 2) were removed

and allowed to reconstitute in tap water. The numbers used were the same as those in the preceding experiment. The data are recorded in Table 5. It will be noted that the controls of this particular lot showed a higher percentage of forms with normal (or enlarged) heads and inhibited tails than did those of the previous lot. The pieces from animals treated with pilocarpine before section showed a *decrease* in number of forms with inhibited tails. This decrease was partly obscured by the large number of deaths, but, if the proportion of forms with normal heads and inhibited tails (Table 5, I*a*) is compared with those with normal heads and normal tails (Table 5, I*b*), the decrease is obvious at both levels (control *A*, 66 per cent I*a* to 20 per cent *Ib;* test *A*, 44 per cent *Ia* to 20 per cent *Ib;* control *B*, 40 per cent *Ia* to 34 per cent I*b;* test *B*, 22 per cent I*a* to 24 per cent I*b*).

TABLE 4

EFFECTS OF PILOCARPINE WHEN APPLIED AFTER SECTION ON THE RECONSTITUTION OF SHORT (SIXTEENTHS) ANTERIOR PIECES OF *D. dorotocephala*
(Values Given in Percentages)

Experiments	Head Types*						Apolar	Bipolar	Dead
	Ia	Ib	II	III	IV	V			
A, tap-water control	50	34	0	0	6	2	1	0	6
B, tap-water control	24	32	0	1	12	10	16	0	4
A, M/1,000 pilocarpine; first 30 hours after section	70	14	0	0	0	0	0	0	16
B, M/1,000 pilocarpine; first 30 hours after section	48	20	2	0	2	0	2	0	26

* Ia—normal heads, inhibited tails; Ib—normal heads, normal tails; II—teratophthalmic, normal tails; III—teratomorphic, normal tails; IV—anophthalmic, normal tails; V—acephalic, normal tails.

TABLE 5

EFFECTS OF PILOCARPINE WHEN APPLIED BEFORE SECTION ON THE RECONSTITUTION OF SHORT (SIXTEENTHS) ANTERIOR PIECES OF *D. dorotocephala*
(Values Given in Percentages)

Experiments	Head Types*						Apolar	Bipolar	Dead
	Ia	Ib	II	III	IV	V			
A, tap-water control	66	20	0	0	0	0	4	0	10
B, tap-water control	40	34	0	0	14	2	4	0	6
A, M/1,000 pilocarpine, 5 days before section	44	20	0	0	0	0	0	0	36
B, M/1,000 pilocarpine, 5 days before section	22	24	2	0	6	6	6	0	34

* For explanation of head types see note to Table 4.

III. EFFECT OF PILOCARPINE ON SHORT POSTERIOR PIECES IN WHICH POSTERIOR SECTION WAS DELAYED FOR 24 HOURS

Six hundred worms were used in these experiments, which involved one control and five tests. The experiments were first performed with 50 pieces to the control and each test lot. The work was then

repeated with the same numbers, and, since the data from the two separate experiments were almost identical, they were not separated. All pieces were approximately one-sixteenth the length of the intact animal and from the region just posterior to the mouth (Fig. 2, level *I*). Posterior section was delayed 24 hours in all. The test agent was always M/1,000 pilocarpine.

TABLE 6

EFFECTS OF M/1,000 PILOCARPINE ON SHORT POSTERIOR PIECES (I-LEVELS) IN WHICH ANTERIOR SECTION OF PIECE WAS MADE 24 HOURS BEFORE POSTERIOR SECTION (Pieces One-sixteenth the Length of the Whole Animal)

Experiments	Head Types*						Apolar	Bipolar	Dead
	Ia	Ib	II	III	IV	V			
1. Tap-water control	38	51	2	0	0	0	0	6	3
2. Pieces in pilocarpine for 48 hours after posterior section	19	34	3	0	0	0	0	38	6
3. Pieces in pilocarpine for 24-hour interval between anterior and posterior section	10	72	2	0	0	0	0	5	11
4. Pieces in pilocarpine from moment of anterior section to 48 hours after posterior section (72 hours)	16	51	9	0	0	0	0	5	19
5. Parent-animal in pilocarpine for 5 days before anterior section	12	62	10	4	0	0	0	0	12
6. Parent-animal in pilocarpine for 5 days before anterior section and pieces in pilocarpine for 24-hour interval between anterior and posterior sections	6	40	18	1	1	1	0	0	33

* For explanation of head types see note to Table 4.

It will be noted (Table 6, 1) that the control gave almost all normal heads from these short pieces. Also to be noted was the fact that 38 per cent had inhibited tails and 6 per cent had developed heads at both ends (bipolars). When the pieces were subjected to pilocarpine for 48 hours after posterior section and then returned to tap water (Table 6, 2) the number of forms with normal heads and inhibited (or normal) tails was decreased, but the number of bipolar forms was markedly increased (38 per cent of total).

Pieces in pilocarpine for the 24-hour interval between anterior and posterior section (Table 6, 3) showed a definite increase in forms with normal tails (decreased scale of organization), with the proportion of bipolars being little affected. Pieces in pilocarpine for 72 hours (from the time of anterior section to 48 hours after posterior section [Table 6, 4]) showed a decrease in forms with inhibited tails and little change in the numbers of bipolars. The proportions in this experiment were disturbed somewhat by the high percentage (19) of deaths.

When the intact animals were treated with pilocarpine before section (Table 6, 5), the percentage of forms with inhibited tails was decreased and those with inhibited heads increased. This was even more noticeable when the period of exposure was extended to cover the 24-hour interval between anterior and posterior section (Table 6, 6), even though the number of deaths was greatly increased. It should also be noted that treatment with pilocarpine before sec-

tion completely eliminated bipolarity in these short pieces.

These experiments with 24-hour delay of posterior section in short posterior pieces show that exposure to pilocarpine (1) before section, (2) during the interval between anterior and posterior sections, and (3) during the interval between sections and for 48 hours after posterior section *decreases* the scale of organization in the pieces (head forms on a smaller scale, leaving material for tail formation). The data also show that when pilocarpine is administered for only 48 hours after posterior section, the number of forms with normal tails is decreased, with the majority of the pieces developing either a head or an inhibited tail at the posterior cut surface. This indicates an increase in scale of organization.

IV. EFFECT OF PILOCARPINE ON HEAD FORMATION IN POSTERIOR HALVES

Although evidence of the inhibiting action of pilocarpine was at hand from preceding experiments, it seemed necessary to test the direct effects of the agent on the regeneration of new tissue that was free from the influence of posterior section.

Fifty posterior halves (actually, approximately seven-sixteenths; Fig. 2, piece *X*) were placed in M/1,000 pilocarpine with the same number being placed in the tap-water control. At the end of 7 days at laboratory temperatures (approximately 70° F.), the amount and degree of head regeneration in test and control pieces were carefully noted and compared.

In all cases the amount of regeneration as indicated by the amount of new (unpigmented) tissue was greater in the controls than in pilocarpine. In the controls all had new, well-shaped heads, with rostral regions and auricles formed of new tissue. In the tests, 46 pieces had survived and regenerated new heads. Of these, however, 19 had not yet regenerated visible auricles, 12 had reconstituted auricles in old (pigmented) tissue, with only the remaining 15 having developed auricles (and these smaller than the controls) from unpigmented tissue. All pieces from both tests and controls had developed two eyespots, but here also the inhibitory action of the agent was shown, since the eyespots of the tests were smaller than those of the controls.

DISCUSSION

A number of investigators have shown a gradient in head formation in short transverse pieces from the first zoöid of *Dugesia*. It has been found that the most anterior pieces form almost 100 per cent normal heads, with the number of normals decreasing and the number of inhibited head types increasing as the level of the piece is pushed farther and farther posteriorly in the anterior zoöid. At the most posterior levels it is not unusual to find all pieces from a particular lot developing into headless (acephalic) forms. This phenomenon may be interpreted on the basis of the physiological-gradient theory (see Child, 1941), but there are many facets to the problem which need further elaboration.

All levels of *Dugesia* will form a normal head at the anterior cut surface of a posterior piece if only a single section is made. However, if the cut is made at an anterior level, the head develops more rapidly than if the cut is made more posteriorly. In other words, there is a gradient in *rate* of head formation (Watanabe, 1935*a*). Short pieces (with anterior and posterior cut surfaces) from posterior regions of the first zoöid form few, if any, normal heads, apparently because of factors imposed upon the head-forming region by posterior section. That is, head-inhibiting factors arise at the posterior

cut surface. These factors are thought to extend forward from their site of origin by way of the ventral nerve cords (Buchanan, 1922; Child and Watanabe, 1935; Watanabe, 1935*b*). Presumably, a head develops on a short transverse piece only if the head-forming region of that piece can escape the inhibitory influence from the posterior cut surface. It seems that the reason that short transverse pieces from anterior levels form a high percentage of normal heads is because these pieces, being from a high gradient level where the head-forming cells are more active, are able to escape from posterior inhibitory factors. This is not the case with short pieces from more posterior levels.

Many agents have been used in the modification of head frequency (see Child, 1941, pp. 183–90, for review). It seems that head frequency may be increased by stimulating the cells at the anterior cut surface of a piece or by blocking in some way the posterior inhibitory factor. Likewise, head frequency may be decreased through the inhibition of the head-forming cells or by increasing the posterior effects.

The work reported in the present paper shows that pilocarpine (M/1,000) definitely inhibits the growth of new (head-forming) tissue at the anterior cut surface. It was evident from the head-frequency experiments with one-eighth pieces and the scale-of-organization experiments with one-sixteenth pieces that pilocarpine caused an increase in head frequency and scale because of differential action on head-forming tissue and on the posterior inhibiting factor. That is, on direct exposure, pilocarpine, while inhibiting the head-forming cells, inhibited or blocked posterior effects to a greater extent. When the intact animals (but not the pieces from these animals) were treated with pilocarpine, head frequency as well as scale of organization decreased. These data indicate that pilocarpine may act on the presumptive head-forming cells of the still intact animal so that, when section is made, they (the head-forming cells) are not readily mobilized. Added to this is the inhibitory action of the posterior cut surface which, appears only after section and is therefore less subject to inhibition or block by the agent.

It has been shown (Rulon and Child, 1937) that the inhibitory agent KCN, when applied to short anterior transverse pieces, will cause a decrease in the scale of organization. This is shown by a decrease in the percentage of forms with inhibited tails and an increase in forms with normal tails. This decrease in scale may be interpreted as due to the direct inhibition of head-forming cells, which results in a smaller head with less in the way of physiological dominance. The present experiments show quite definitely that pilocarpine, after section, causes an *increase* in scale of organization in short anterior pieces. It seems that conditions within the piece are so affected (probably by the differential inhibition of the posterior inhibitory factor) that the bulk of available cells may be used in forming a head (of a scale too large for the size of the piece), while tails do not form because the gradient now determined is so much longer than the piece that conditions for tail development do not appear. It is also shown that, if the pilocarpine is used before section, certain relationships in the still intact animal are modified so that the head-forming cells in the newly sectioned pieces (together with possibly less inhibition of posterior factors) form heads on a smaller scale. This results in a shorter gradient with a greater proportion of the piece for tail formation.

Although short transverse pieces from

the posterior levels normally reconstitute into a high percentage of forms with inhibited heads, it was noted by Child and Watanabe (1935) that, following a few hours' delay of posterior section, these pieces would develop 100 per cent normal heads. F. S. Miller (1937) found that strychnine applied to pieces from posterior levels caused a high percentage of bipolar forms and that the percentage was markedly increased if there were a few hours' delay in making the posterior section. In the present experiments it was evident that when a single cut was made at the level just posterior to the mouth, a new head of a size characteristic of the posterior half of the animal began to form. When a second cut (one-sixteenth the length of the entire animal behind the first cut) was made 24 hours later, head-forming cells had already become activated to form a head too large for the piece. Under such conditions a gradient was determined for a much longer piece—a condition which prevented, to a certain extent, the appearance of tail-forming factors. When pilocarpine was used before posterior section, however, the head-forming cells were held partly in check. This resulted in a shortened gradient system, with greater number of tails being formed. When pilocarpine was used after posterior section, only a few tails developed. Instead, 38 per cent of the pieces developed with heads at both ends. This appeared to be the result of a loss of correlating factors in pieces that were primed for a much increased scale of organization and had increased in head-forming cells. The level of the pieces (which was not far from the fission plane and therefore possessed but little differential between anterior and posterior cut surfaces) was also probably an important contributing factor.

SUMMARY

1. When transverse pieces (one-eighth the length of the whole 14–16-mm. animal) were exposed to certain concentrations (M/500–M/15,000) of pilocarpine, the head frequencies of the reconstituting forms were increased; but, when the parent-animals were exposed to the agent (M/500–M/1,000) and the pieces were allowed to reconstitute in tap water, the head frequencies were decreased (in 7 out of 8 cases).

2. When short transverse pieces (one-sixteenths) from the level just posterior to the head were treated with pilocarpine (M/1,000), the scale of organization was increased; but, when the parent-animals were treated before section, the scale was decreased.

3. Short posterior pieces (one-sixteenths) with 24 hours' delay of posterior section showed a decrease in scale of organization when treated with M/1,000 pilocarpine before section, during the interval between anterior and posterior section, and during the interval between sections plus 48 hours after posterior section.

4. Similar short posterior pieces, when treated with pilocarpine for only 48 hours after posterior section, showed an increased scale of organization, as well as a loss of correlative factors, as evidenced by the development of a high percentage of bipolar forms.

5. Posterior halves reconstituting in M/1,000 pilocarpine develop heads more slowly and with less new tissue than those reconstituting in tap water.

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THE MODIFICATION OF DEVELOPMENTAL PATTERN IN THE SAND DOLLAR BY MALONIC ACID[1]

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WHEN the newly fertilized eggs and late-cleavage stages of *Arbacia* were exposed to certain concentrations of malonic acid, cleavage and gastrulation were retarded (Rulon, 1948). Development, however, usually continued and gave gastrulae or plutei with greatly inhibited basal regions but with apical regions much enlarged into highly active ciliated knobs. In *Arbacia* this agent was not effective in causing exogastrulation or entodermization.

Since the eggs of *Dendraster* have proved to be excellent material for studies on the experimental modification of development and since the author has recently started a program (Rulon, 1949) dealing with the effects of various enzyme inhibitors on developmental pattern in the eggs of this sand dollar, it seemed advisable to determine the effects of malonic acid (a reputed inhibitor of succinic dehydrogenase) on this egg and to compare its effects with those caused by other inhibitors.

[1] The writer is most grateful to the Graduate School of Northwestern University for material support given this work. To Dr. L. R. Blinks, director of the Hopkins Marine Station, Pacific Grove, California, who provided the facilities for this work, the writer is much indebted.

MATERIAL AND METHODS

The sand dollars (*Dendraster excentricus*) were dredged from Monterey Bay during the breeding season and kept until used in running sea water in the aquarium room of the Hopkins Marine Station. After being washed in tap water, the animals were opened with scissors, and only the eggs exuding from ripe, red ovaries of mature females were taken. These eggs were drawn up in thick masses into medicine droppers and transferred to sea water in finger bowls. After several washings the eggs were fertilized by the addition of a few drops of sperm suspension.

Newly fertilized eggs and eggs in various stages of development were transferred to the test solutions and sea-water controls with medicine droppers. All experiments were run at laboratory temperature, which varied during the course of these experiments by no more than 1° from 18° C. Observations were made at regular intervals of both test and control forms.

A number of preliminary experiments were run to determine the concentration range over which the test agent was effective. This was accomplished by adding graded quantities of stock solution (made up, first, at iso-osmotic strength and then diluted with sea water) of malonic acid to 100 cc. of sea water. Fertilized eggs were then added to the different test solutions and were followed through development.

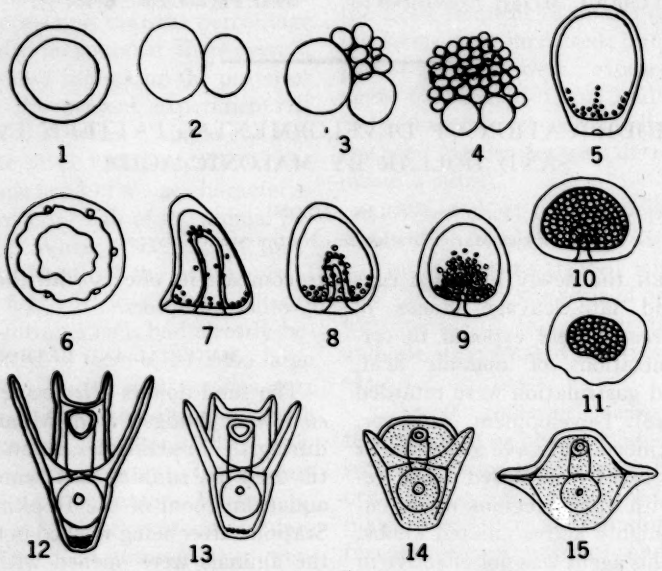
EXPERIMENTAL

I. EFFECTS OF MALONIC ACID WITH EXPOSURE BEGINNING AFTER FERTILIZATION

Newly fertilized eggs of *Dendraster* were placed in the following solutions:

1..... Normal sea water (control)
2..... 100 cc. sea water + 2.0 cc. 0.036 M malonic acid
3..... 100 cc. sea water + 2.5 cc. 0.036 M malonic acid
4..... 100 cc. sea water + 3.0 cc. 0.036 M malonic acid
5..... 100 cc. sea water + 3.5 cc. 0.036 M malonic acid

After 6 hours, when the control eggs had developed into normal young blastulae (Fig. 1), those in solution *2*, although having reached early blastula stages, were definitely retarded. In solution *3* the inhibition was so great that only unequal cleavage stages of from 2 to 64 cells (Figs. 2–4) were to be found. Eggs in solution *4* had advanced no further than 2-, 3-, and 4-cell stages, with the blastomeres unequal in size and the cleavage pattern irregular. No cleavage had taken place in eggs in solution *5* by this time.



FIGS. 1–15.—Fig. 1, normal 6-hour blastula. Figs. 2–4, eggs that have been in 100 cc. of sea water plus 2.5 cc. of 0.036 M malonic acid for the first 6 hours of development. Fig. 5, normal 12-hour early gastrula. Fig. 6, inhibited blastula after first 12 hours of development in 100 cc. of sea water plus 2.5 cc. of 0.036 M malonic acid. Fig. 7, normal 24-hour prepluteus. Figs. 8–11, 24-hour inhibited larvae that have been in 100 cc. of sea water plus 2 cc. of 0.036 M malonic acid since fertilization. Fig. 12, normal 55-hour pluteus. Fig. 13, 55-hour pluteus that has developed under continuous exposure to 100 cc. of sea water plus 2 cc. of 0.036 M malonic acid. Figs. 14–15, 55-hour plutei that have developed under continuous exposure to 100 cc. of sea water plus 2.5 cc. of 0.036 M malonic acid.

By 12 hours the eggs developing in normal sea water were free-swimming early gastrulae, with primary mesenchyme and often the beginnings of invagination (Fig. 5). Those in solution *2* showed the inhibitory effect of the agent by having slightly thicker walls and less mesenchyme. In solution *3* most of the eggs had developed into thick-walled, irregular, slow-moving blastulae, many of which were still in their fertilization membranes (Fig. 6). Many were losing cells to the outside, and some had become completely dissociated. In solutions *4* and *5* the eggs were no more advanced than when the 6-hour observations were made.

At 24 hours the controls were free-swimming, bilateral preplutei (Fig. 7), while the eggs placed in solution *2* had developed into larvae ranging all the way from slightly inhibited, free-swimming, bilateral gastrulae (Figs. 8, 9) to grossly inhibited, thick-walled, cell-filled forms (Figs. 10, 11). In solution *3* many had cytolyzed, while those still alive were practically all cell-filled, bottom, radial, or irregular forms, with only a few showing traces of invagination. The eggs in solution *4* were still in early cleavage, while a few in solution *5* showed a trace of abortive cleavage.

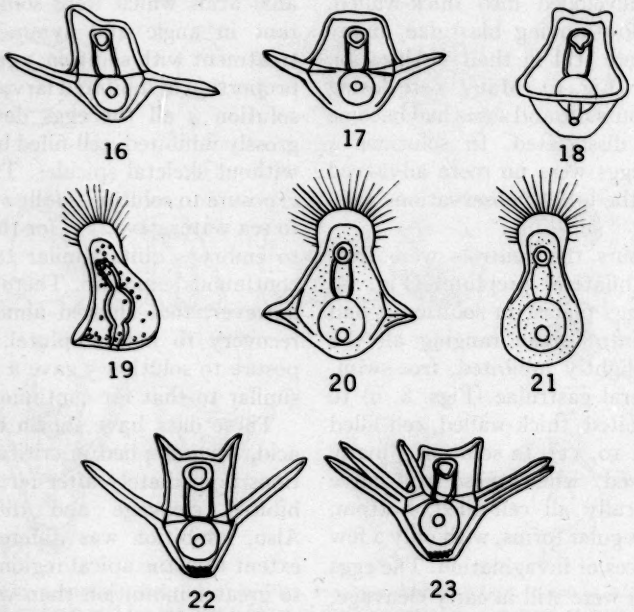
Fifty-five hours after the beginning of the experiment the controls had advanced to well-developed plutei with long oral and anal arms (Fig. 12). Although a few of those in solution *2* were grossly inhibited, cell-filled bottom forms, most were free-swimming, though inhibited, plutei (Fig. 13). Approximately half the eggs in solution *3* gave rise to slow-moving, granular plutei, with undifferentiated oral lobes and short anal arms (Fig. 14), often at increased angles (Fig. 15), but the remainder developed into grossly inhibited, irregular, cell-filled bottom forms. Eggs in solutions *4* and *5* never developed past the abortive and irregular cleavage stages.

When eggs were returned to sea water after 6 hours' exposure to solution *2*, normal plutei usually developed, although there were a few grossly inhibited forms and forms with inhibited oral lobes and anal arms which were sometimes aberrant in angle and symmetry. Similar treatment with solution *3* gave a greater proportion of inhibited larvae, while with solution *4* all the eggs developed into grossly inhibited, cell-filled bottom forms without skeletal spicules. Twelve hours' exposure to solution *2* followed by return to sea water gave rise, for the most part, to embryos quite similar to those with continuous exposure. There were a few, however, that showed almost complete recovery to normal plutei. Similar exposure to solution *3* gave a picture very similar to that for continuous exposure.

These data have shown that malonic acid, when applied in critical concentrations immediately after fertilization, inhibited cleavage and differentiation. Also, inhibition was differential to the extent that the apical region was subject to greater inhibition than was the basal region. The increase in angle of anal arms in solution *3* (Fig. 15) may have indicated some degree of differential tolerance, or it may have meant inhibition of ventrodorsality, in that the ventral area, instead of becoming deeply concave as in a normal pluteus, remained convex or only slightly flattened. There was a certain degree of recovery when the eggs were returned to sea water after a short time exposure to malonic acid. For the most part this recovery was general (proportionate) rather than differential (disproportionate). In these experiments malonic acid failed to cause exogastrulation or entodermization—modifications often found with inhibitory agents.

II. EFFECT OF MALONIC ACID WITH EXPOSURE BEGINNING AT THE MID-BLASTULA STAGE

When 6-hour blastulae were exposed continuously to solution *2*, almost all developed into plutei with anal arms at increased angles and commonly asymmetrical (Fig. 16). The oral lobes were and very little, if any, skeleton (Figs. 18–21). Anal arms, when present, were very short and at increased angles. The ventral surface was commonly much flattened or even convex. Oral arms did not appear, and in many the apical end became greatly enlarged into a knob with



FIGS. 16–23.—Fig. 16, larva resulting from continuous exposure of blastula to 100 cc. of sea water plus 2 cc. of 0.036 M malonic acid. Fig. 17, larva resulting from continuous exposure of blastula to 100 cc. of sea water plus 2.5 cc. of 0.036 M malonic acid. Figs. 18–21, larvae resulting from continuous exposure of blastulae to 100 cc. of sea water plus 3 cc. of 0.036 M malonic acid. Figs. 22–23, larvae resulting from the exposure of blastulae to 100 cc. of sea water plus 2 cc. of 0.036 M malonic acid for 7½ hours and then returned to sea water.

usually not well differentiated and also commonly asymmetrical. Continuous exposure to solution *3*, beginning at this stage, gave plutei with shorter anal arms and less differentiated oral lobes. The angles of the anal arms, however, were much increased (Fig. 17), and the arms were commonly asymmetrical and duplicated. In solution *4* the blastulae developed into free-swimming and bottom larvae with an excess of mesenchyme highly active cilia (Figs. 19–21). By 27 hours, blastulae in solution *5* had become dissociated into their separate cells while still within their fertilization membranes.

When the blastulae were returned to normal sea water after 7½ hours' exposure to solution *2*, most developed into plutei with differentiated and enlarged oral lobes and long anal arms at increased angles (Fig. 22). In a few the anal arms

were duplicated (Fig. 23), asymmetrical, or askew. There was also an occasional grossly inhibited form with an enlarged, ciliated, apical knob. Similar exposure to solutions *3* and *4* resulted in a large number of grossly inhibited forms at the bottom of the cultures. Most developed an excess of mesenchyme, and many had asymmetries and duplications in anal arms. Despite obvious inhibition by this treatment, many had widened oral lobes and anal arms at increased angles. On the whole, the degree of inhibition was much less than that shown by continuous exposure to these solutions.

In general, it has been shown by these experiments that exposure, beginning at the blastula stage, to weak solutions of malonic acid caused a definite increase in the angles of the anal arms, although differentiation of the oral lobe was inhibited. With increased concentrations the anal arms became more and more retarded, and the oral lobe, although relatively undifferentiated, became greatly thickened, enlarged, and with the apical tuft of cilia greatly extended. This increase in size of apical end at the expense of the basal may be interpreted as due to the phenomenon of differential conditioning. That is, the more active region in the polar gradient became conditioned more rapidly and therefore enlarged at the expense of the less active basal region. The return to normal sea water after a short time exposure to the malonic acid gave all degrees of differential inhibition, conditioning, and recovery. As in the case of exposure of the one-cell stage, malonic acid failed to cause exogastrulation at the blastula stage.

DISCUSSION

A comparison of the effects of certain enzyme inhibitors on sand-dollar development is of interest. Maleic acid, which is primarily an inhibitor of succinic dehydrogenase and glyoxalase, will (at the blastula stage) often cause the development of differentiated plutei of radial symmetry, as well as a great increase in ventral area of those with bilateral symmetry. It also causes, under certain conditions, the development of thickened, enlarged, apical knobs with highly active cilia (Rulon, 1949). In the present work with malonic acid (also an inhibitor of succinic dehydrogenase) it is shown that exposure at the blastula stage also tends to increase the ventral area and, under slightly more severe inhibition, to cause the development of enlarged apical knobs. Similar apical knobs have been caused to develop in *Arbacia* with malonic acid (Rulon, 1948), ethyl alcohol, and $CuSO_4$ (Child, 1941*a*, pp. 206–7). Sodium azide (Rulon, 1950*a*) and thiourea (Rulon, 1950*b*) do not readily produce such modifications. Azide, an inhibitor of cytochrome oxidase, catalase, peroxidase, and other iron-porphyrin enzymes, as well as phosphorylations, protein synthesis, etc., is a most effective agent in the production of exogastrulation, often with extensive entodermization. Thiourea, an inhibitor of oxidative enzymes other than cytochrome oxidase, also causes exogastrulation and entodermization under certain conditions, but not to the degree or extent of azide (approximately 20 per cent with thiourea and 90 per cent with azide). Maleic and malonic acids are both ineffective in causing exogastrulation and entodermization. Azide and thiourea are both good inhibitors of apical and ventral regions, but again azide is more effective than thiourea.

Apical and ventral regions (those most active in the apicobasal and ventrodorsal physiological gradients of the blastula) have both been shown to have high cytochrome oxidase (indophenol oxidase) activity in the blastula stage (Child,

1941*b*). There are also indications that cytochrome oxidase activity may in some way be associated with the invagination reaction at the basal region of the late blastula. It may be that agents which differentially inhibit apical and ventral regions, as well as the invagination reaction, do so, directly or indirectly, by inhibiting cytochrome oxidase. But this is certainly not the whole explanation. Thiourea may inhibit oxidative enzymes other than cytochrome oxidase (as found by Lerner and Chaikoff [1945] in thyroid tissue). Also, normal gastrulation may be largely dependent on phosphorylations (as found by Barth and Jaeger [1947] in amphibian development). Azide is a good inhibitor of phosphorylations (Loomis and Lipmann, 1949), and the stimulus to exogastrulation (at least by this agent) may well be in the inhibition, at the critical moment, of activities other than cytochrome oxidase. The fact that agents such as azide, cyanide, thiourea, lithium, thiocyanate, pilocarpine, crowding, tobacco smoke, etc.,[2] all produce varying degrees of the same modifications certainly indicates that the problem of differential inhibition is not simple and may be largely quantitative in nature.

The actions of malonic acid, maleic acid, azide, and thiourea are similar in many respects. All, at certain critical concentrations, inhibit cleavage, affect blastulae less than newly fertilized eggs, cause an excess of mesenchyme, and inhibit differentiation of the oral lobe, anal arm and skeletal development, and the development of ventrodorsality. Yet neither malonic nor maleic acid is effective in producing exogastrulation. From this it would appear that the activities[3] inhibited by these agents have little to do with the invagination process.

Blastulae in slightly inhibitory concentrations of malonic and maleic acids tend to develop into plutei with increased ventral areas and with anal arms at increased angles (although the oral lobes are commonly inhibited). With more severe inhibition the basal regions of the larva may be strongly inhibited, while the apical region develops into an enlarged ciliated knob (Figs. 19–21, for malonic acid). These modifications have been interpreted (Rulon, 1949, for maleic in *Dendraster;* Rulon, 1948, for malonic in *Arbacia*) as the result of differential conditioning (differential acclimation, differential acquirement of tolerance). In the case of increased ventral surfaces and increased angles to anal arms, it seems that the more active ventral region conditions more readily to the agent than does the less active dorsal region in the ventrodorsal gradient system and thereby increases in size at the expense of the dorsal. The ventral region may also show differential recovery (because of its higher activity) when returned to sea water after short time exposure (Fig. 22 for malonic acid). The apical region may increase in size over the basal region through differential conditioning because of its high position in the apicobasal gradient, even though differentiation in the form of skeleton, etc., is definitely inhibited. This is in accord with the concept of quantitative graded differentials as being the primary developmental patterns.

Since malonic acid primarily inhibits

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[2] See Child, 1940, 1948; Rulon, 1941*a*, *b*, 1946, 1950*a*, *b*, for data and references.

[3] Malonic acid is known to inhibit succinic dehydrogenase, while maleic acid inhibits glyoxalase, as well as succinic dehydrogenase. Since the presence of these enzymes has not been determined in the eggs of *Dendraster*, it cannot be stated with certainty that one is dealing with their inhibition. Even if these enzymes are proved to be present, it is not unlikely that malonic and maleic acid have (either directly or indirectly) other effects on metabolic processes.

succinic dehydrogenase (and it may also inhibit other processes), it has been stated (Rulon, 1948) that a gradient of succinic dehydrogenase activity which corresponds with the physiological gradient pattern (see Child, 1941*a*) may exist. This may well be the case, but further study has given an alternative explanation. Maleic and malonic acids may not inhibit certain prominent enzyme activities (cytochrome oxidase, other oxidative enzyme, perhaps phosphorylase, etc.) in the apical and ventral regions (just as they may fail, under certain conditions, to inhibit such activities at the site of invagination) so greatly as they do other enzyme (succinic dehydrogenase, glyoxalase) activities. Therefore, under malonic and maleic acid inhibition, apical and ventral regions should have higher physiological activity and, under certain conditions, increase in size at the expense of less active, more inhibited regions. With this explanation the apparent differential conditioning is actually *less inhibition* of apical and ventral regions.

SUMMARY

1. In critical concentrations malonic acid inhibited cleavage and differentiation in the developing eggs of *D. excentricus*.

2. When the agent was applied at the one-cell stage, the apical regions were subject to greater inhibition than were the basal regions, and the angles of the anal arms of the inhibited plutei tended to increase.

3. Blastulae (6-hour) were able to tolerate and undergo a certain degree of differentiation in concentrations of malonic acid that were lethal to the cleaving egg.

4. In low concentrations the blastulae developed into plutei with increased angles of anal arms, although differentiation of oral lobes was inhibited.

5. With higher concentrations, anal arms and basal regions were, in general, more inhibited, while apical regions, although relatively undifferentiated, became greatly enlarged, thickened, and with apical ciliary tuft greatly extended.

6. When blastulae were returned to normal sea water after a short time exposure to low concentrations of malonic acid, differential recovery resulted in enlarged differentiated oral lobes and long anal arms at increased angles.

7. Malonic acid was ineffective in causing exogastrulation or entodermization under the conditions of these experiments.

8. The comparative effects of malonic acid, maleic acid, sodium azide, and thiourea on sand-dollar development are discussed, and a tentative interpretation, based on relative enzyme inhibition, is given.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY, VOL. XIII: *BIOLOGICAL APPLICATIONS OF TRACER ELEMENTS*[1]

Reviewed by DR. ALFRED MARSHAK
New York University, College of Medicine

UNLIKE previous symposia of this series, the conference represented by this volume is devoted to discussion of method, i.e., the use of tracers in biological materials, emphasis being on results rather than on techniques. Although a few of the papers deal with design and interpretation of experiments with tracers, most are descriptions of results obtained. To a limited extent the volume represents a review of achievement in this field.

A first impression of the book is one of bewilderment since almost completely unrelated papers appear in juxtaposition. For example, the first is on chromosome breakage with P^{32}, the second on photosynthesis, the third on the use of O^{18}. The historical sketch of the biological application of tracers in biology begins on page 129. With such an arrangement, it is well-nigh impossible to get any impression of the spirit of the meetings or a reasonable concept of what has been accomplished and what needs to be done. As presented, it represents simply a pool of miscellaneous information.

In the Foreword the editor mentions criticism of previous volumes for arranging papers alphabetically by authors. He feels that such arrangement is justified, since the volume will be used primarily for reference. It is difficult to understand this position because the book contains an index both of subject matter and of authors. With such an index, the alphabetical arrangement seems entirely superfluous and serves only to confound, rather than to aid, the reader who wishes to get a survey of the field, and it imposes an unnecessary burden upon the novice, for whom such a volume might form a basis for orientation rather than a grab bag of more or less information.

[1] New York: Biological Laboratory, Cold Spring Harbor, 1948. Pp. 222.

SELECTED INVERTEBRATE TYPES,[1] EDITED BY F. A. BROWN, JR.

Reviewed by RALPH BUCHSBAUM
University of Pittsburgh

TEACHERS will welcome a new laboratory manual for invertebrate zoölogy. Most American manuals carefully avoid presentation of many facts and drawings, on the theory that a manual should be no more than a guide to laboratory study, from which the student will derive his own information and drawings. *Selected Invertebrate Types* differs from such manuals in providing considerable information and illustrative material for each species treated. There are 235 line drawings, most of them executed by Miss Marie Wilson, in a uniform, attractive, and clear style. Nearly all the labeling is direct, although a few diagrams, for no obvious reason, have numbers instead of words, the numbers referring to words in the legend or to words in the text. About 42 per cent of the drawings are original. The Table of Contents is a directory of the forms discussed and, at the same time, a table of phyla, subphyla, classes, subclasses, orders, and species. Page numbers refer to the location of the description of each of the

[1] New York: John Wiley & Sons, 1950. Pp. 597 +235 figs. $6.00.

129 species included, the account of which makes up the content of the volume.

The Bibliography is classified phylogenetically. It has a general section of 14 titles and a section by phylum and species of 475 well-selected and recent references. There is a short but adequate index of scientific and common names of the species. The book contains no specific directions for student records, this being left to individual teachers to provide.

The contributors to this volume are: F. A. Brown, Jr. (Northwestern University), who is also the editor, W. Balamuth (Northwestern University), J. B. Buck (National Institute of Health), W. D. Burbanck (Drury College), C. G. Goodchild (S.W. Missouri State College), Libbie H. Hyman (American Museum of Natural History), Margaret L. Keister (National Institute of Health), L. H. Kleinholz (Reed College), J. H. Lochhead (University of Vermont), Madeline E. Pierce (Vassar College), W. M. Reid (Monmouth College), Mary D. Rogick (College of New Rochelle), and T. H. Waterman (Yale University). Most of the contributors are, or have been at some time, on the staff of the Department of Invertebrate Zoölogy of the Marine Biological Laboratory at Woods Hole, Massachusetts.

Few teachers of zoölogy would deny editor Frank Brown's contention, in the Preface to this manual, that the subject of invertebrate morphology must, of necessity, be compressed more and more in the average college curriculum. Students are no brighter than they used to be, nor are college terms any longer. There is no practicable way of providing for the steady expansion in the curriculum of the newer biological fields without cutting to one-half or one-fourth the time allowed an earlier generation of students for the study of comparative morphology. Those teachers who would argue that morphology is the foundation and the backbone of zoölogy are probably entirely right. But they were long ago answered by Julian Huxley, when he observed that "we do not live in the foundations of our houses, nor are they larger than the superstructure . . . and as for backbones . . . an animal whose backbone weighed more than its muscles, nervous system, and viscera combined, would be biologically very inefficient."

There have been many approaches to the solution of this problem. The trend seems to be the constant pruning of the materials to eliminate as much detail and terminology as possible, while leaving the basic principles. Shorter, better-written textbooks are a part of this approach. In the laboratory there must also be some change from the effective, but time-consuming, method of treating each animal as if it were a small research problem. Editor Brown and his group have attacked this situation by providing a manual in which many facts are presented to the student so that he can more rapidly obtain whatever his teacher considers a necessary foundation.

The editor of the manual estimates that the content of the book is "adequate for a 2-year laboratory course." This provides plenty of leeway for a discriminating selection of types by each teacher for either a one-year or a one-semester course. The reviewer feels that, on the whole, each species is described adequately for laboratory purposes, though there are points at which students will wish for more explicit directions in finding certain structures.

An attempt to estimate the distribution of the text materials by counting the number of lines of text devoted to taxonomy, to morphology, and so on for each of a number of species chosen at random gave the following result: morphology, 46 per cent; laboratory directions, 20; reproduction and embryology, 13; behavior notes, 7; taxonomy, 5; methods of cultivation of the species in the laboratory, 3; physiology, 3; and ecology, 3. Some teachers may wish that certain of the materials included had been left to the textbooks, so that there might have been more room for directing the attention of the student to problems of physiology and behavior. Perhaps the authors felt inhibited in trying to present materials that are now available in Welsh and Smith's *Laboratory Exercises in Invertebrate Physiology*.[2]

The editor states his hope that the manual will serve a secondary purpose in introducing many investigators to invertebrate species suitable for experimental problems in biology and medicine. This reviewer's own experience in trying to advise nonzoölogists on the selection of suitable invertebrate materials suggests that such a manual can serve only as a reference aid for the nonzoölogist. The actual selection of experimental forms had better be made by consultation.

Subsequent editions of the manual will become increasingly useful as the book is tried out in various parts of the country and the experiences of a variety of teachers become incorporated into the presentation. The authors deserve the thanks of zoölogists for producing a volume which contains so much sound material as to serve them at a time when teachers as well as students need efficient teaching and reference aids.

[2] Minneapolis, Minn.: Burgess Pub. Co., 1949.

METABOLISM AND FUNCTION: A COLLECTION OF PAPERS DEDICATED TO OTTO MEYERHOF ON THE OCCASION OF HIS 65TH BIRTHDAY

EDITED BY D. NACHMANSOHN[1]

Reviewed by WARREN H. YUDKIN
Harvard University

PATTERNS of thought and consequently patterns of investigation are taken much for granted by the present generation of biochemists. To remind us neophytes that the patterns we fall into are part of the evolution of what began as truly pioneer thinking becomes a cardinal function of a collection of papers in honor of an outstanding pathfinder. Otto Meyerhof is a pioneer in biochemistry, and the papers in this collection by his colleagues and students display the fruitful results of the spread of his influence into the manifold of biochemistry.

This volume has been divided, roughly, into three parts. Part I, entitled "Muscle," has some comprehensive reviews on the properties, functions, and interactions of muscle proteins by M. Dubuisson, D. M. Needham, H. H. Weber, and W. F. H. M. Mommaerts. These are extremely valuable in presenting authoritative and conflicting points of view in the best manner on a subject beset with conflicts. Part II, entitled "Nerve," in part contains research papers by D. Nachmansohn and by M. A. Rothenberg concerning permeability in relation to nerve function. In general, the former paper ably presents Nachmansohn's newer hypothesis on the mode of axonal conduction and synaptic transmission. F. O. Schmitt has written a summary of recent work on the molecular morphology of muscle and nerve. Part III, entitled "Drug Action," is composed of papers on miscellaneous subjects. C. L. Gemmill has very briefly reviewed the effects of pharmacological agents, such as quinine, naphthoquinones, amidines, alloxan, caffeine, mercury compounds, iodine, and anesthetics, on glycolysis. But in only a very few cases does he consider the specific enzyme which may be attacked in the scheme of glycolysis. The review by G. Wald, entitled "The Interconversion of the Retinenes and Vitamins A *in vitro*," is a detailed account of what is known about the enzymatic oxidation-reduction in the retina which interconverts vitamin A and vitamin A aldehyde (retinene) in the visual cycle. The state of these typically fat-soluble molecules in an aqueous system is also discussed here. In his review, entitled "The Biological Incorporation of Purines and Pyrimidines into Nucleosides and Nucleic Acid," H. M. Kalckar points out the apparent discrepancies in the results gained from enzymatic studies and the observations made in intact organisms by the use of isotopes. Whereas liver nucleoside phosphorylase catalyzes the incorporation of only hypoxanthine and guanine into nucleosides, the intact animal excretes as uric acid and allantoin all N^{15} fed to it in the form of these two purines. On the other hand, the intact animal incorporates fed isotopic adenine into nucleic acids, but liver nucleoside phosphorylase does not catalyze the formation of adenosine from adenine.

Although attention here has been brought to the review articles in this volume, they are outnumbered by the papers on original contributions, as, for example, the paper by A. L. Grafflin and S. Ochoa on the partial purification of isocitric dehydrogenase and oxalosuccinic carboxylase, and that by F. Lipmann and L. C. Tuttle on the condensation, catalyzed by lipase, of fatty acids with hydroxylamine. And, indeed, many of these show aptly the gifts which Meyerhof has transmitted to his scientific children and grandchildren. A. V. Hill and A. von Muralt have contributed articles of historical interest which emphasize Meyerhof's contributions to muscle biochemistry and thus serve to give perspective. Six photographs, old and new, of Meyerhof, his former institute, his colleagues, and his students have been appropriately included in this volume. In all, there are thirty-eight articles in this volume, thirteen of which might be called "reviews." The majority of papers are in English, but practically all have summaries in English, French, and German. In a few cases, however, these summaries are inclined to be untrustworthy.

The very method of organizing and issuing a *Festschrift* in these times can easily result in the publication of forced and redundant material which does but token honor. In many papers in this volume one can find honor paid implicitly or explicitly to Otto Meyerhof. And yet, through hardly any fault of the authors involved, there are some papers which are functionless in orienting the reader in his regard for Otto Meyerhof. If the function of a *Festschrift* might be that set forward here, then one wonders whether the present editorial methods should not be more relaxed and spontaneous.

[1] New York: Elsevier Press, 1950. Pp. 348. $7.00.

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